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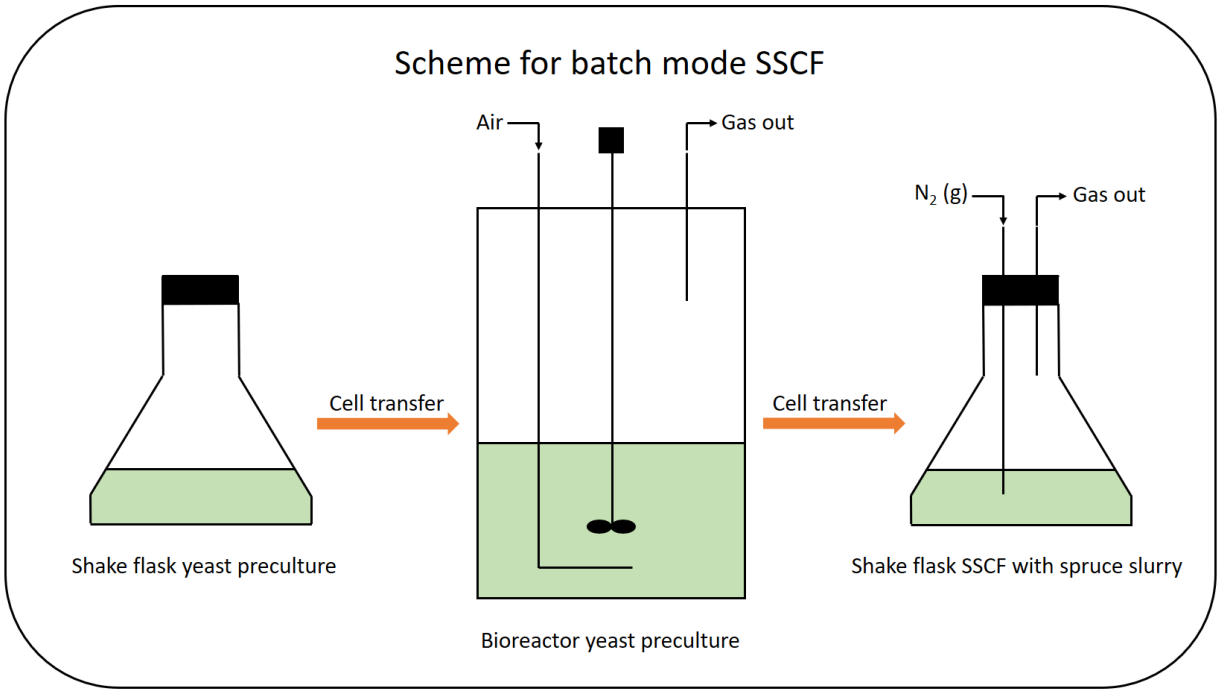

Assessment of fermentability of steam-pretreated spruce tips, needles and branches for bioethanol applications

Master's thesis in Biotechnology

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Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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Cover: Figure of the batch mode simultaneous saccharification and co-fermentation process utilizing pretreated spruce tips, needles and branches.

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Abstract

The production of bioethanol from fermentation-based bioprocesses utilizing lignocelluosic feedstocks is an option for replacing fossil based fuels. By using genetically modified yeasts that co-consume glucose and xylose, it is possible to ferment lingocellulosic materials such as spruce for bioethanol production.

In this project the fermentability of spruce tips, needles and branches, pretreated by acid-catalyzed steam explosion according to a design of experiments plan, was evaluated in terms of ethanol titer, rate and yield as well as cell viability. In order to ferment the spruce tips, needles and branches, sufficient cell concentrations are needed. A preculture method was developed where enough cells were produced and harvested in the same physiological state in different batches for simultaneous saccharification and co-fermentation.

A anaerobic shake flask system was used to ferment the spruce tips, needles and branches. Final ethanol titers of up to 10 g l^{-1}, average volumetric ethanol production rates of up to 0.35 g l^{-1} h^{-1} and final yields of ethanol on available glucose and on available glucose together with xylose of up to 0.19 $g_{Ethanol}$ $g_{Glucose}^{-1}$ and 0.16 $g_{Ethanol}$ $g_{Glucose+Xylose}^{-1}$ respectively were observed. The final cell concentrations, colony forming units and growth rates observed were all fairly low values of up to 3.00 x 10^5 cells ml^{-1}, 1.19 x 10^7 CFU ml^{-1} and 2.36 x 10^{-2} h^{-1} respectively.

Even though there may be some indications on preferable pretreatment conditions in terms of fermentability, more testing and experiments are required to make statistically significant recommendations.However, trends observed in this project points to either high temperature and short time or low temperature and long time as preferable pretreatment process conditions. Materials pretreated under these conditions showed the highest final titers and yields.

Acknowledgements

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Tobias Asp, Gothenburg, June 2018

Contents

1

Introduction

Fossil fuels contribute to the emission of greenhouse gases, leading to global warming, rise in sea level, loss of biodiversity and urban pollution [1]. It has also been estimated that fossil fuels will be exhausted within 40 to 50 years [2]. These are reasons to move on from fossil fuels to reach a more sustainable society. Bioethanol produced from fermentation is an alternative to fossil fuels. If the ethanol production uses only renewable energy, no net CO_2 is added to the atmosphere [3]. This makes bioethanol a potentially environmentally beneficial energy source.

Bioethanol has a octane number of 108. Thereby it prevents early ignition and engine knocking in fuel combustion engines [4]. With its high oxygen content it combusts cleaner compared to petroleum and thereby contributes to lower emissions [4]. These traits enable bioethanol to currently be one of the most industrially produced fossil substitutes with a market of 58 billion dollars annually [5]. Approximately 86000 kton bioethanol per year is produced [5].

Bioethanol can be produced via fermentation of different sugar-based, starchy or lignocellulosic raw materials. Bioethanol production via fermentation of sugar- or starch-based raw materials is referred to as first generation (1G) bioethanol. The most common feedstocks for 1G bioethanol in the last few years are Brazilian sugar cane and US corn [6]. A major drawback with 1G bioethanol is that the feedstock used directly competes with the food and feed industry [6].

Bioethanol produced from lignocellulosic raw materials is a more sustainable option as the feedstocks used are non-edible lignocellulosic whole plant biomass, non-edible residues from food crop production or non-edible residues from forest management [2]. Bioethanol produced from these feedstocks is called second generation (2G) bioethanol. One lignocellulosic material is the softwood spruce [7]. Spruce occupies 18 million ha of forest land in northern Europe and has a total growing stock of 2700 million m^3 which makes it an available feedstock in northern Europe [7]. 2G bioethanol is more sustainable compared to 1G bioethanol production in a direct food versus feed perspective as well as in terms of land usage [8]. The plant biomass used for 2G bioethanol processes can be bred specifically for bioethanol production [8]. This can increase production per unit land area and result in a higher land used efficiency.

1.1 Aim

There are two aims of this project. The first aim is to experimentally determine the fermentability of slurries of spruce tips, needles and branches that have previously been pretreated in 13 different conditions and analyzed in terms of composition. The fermentability is assessed in terms of ethanol yield, ethanol production rate and cell viability. The second aim is to investigate whether it is possible to statistically connect fermentation characteristics with the conditions applied during pretreatment.

1.2 Limitations

The project is limited to fermentations in shake flasks and not any larger scales. The yeast preculture will however be propagated in bioreactors. Sampling will be limited based the available analytic methods. The focus lies on ethanol yield, ethanol production rate and cell viability.

2

Theory

2.1 Lignocellulosic biomass

Lignocellulosic materials include feedstocks such as wood, forestry waste, agricultural residues and municipal solids [9]. Lignocellulosic biomass consists of three major components, cellulose, hemicellulose and lignin [10]. The lignocellulose complex is a matrix of lignin and cellulose bound by hemicellulose chains [10]. Hemicellulose are branched polysaccharides consisting of pentoses such as D-xylose and D-arabinose as well as hexoses such as D-glucose, D-galactose and uronic acids [9]. Lignin is an aromatic polymer of phenolic compounds derived from phenylpropanoid precursors [9]. Cellulose is a polymer of β-1,4-linked D-glucose units [9]. Softwoods such as spruce typically consists of a higher fraction of glucose and mannose units in the hemicellulose compared to hardwood [9]. This results in a higher glucose content and lower xylose content in softwood hydrolysates compared to hardwood hydrolysates.

A major challenge with lignocellulosic materials as feedstock for bioethanol production are the amount of inhibitory compounds released in the process. There are three major groups of inhibitors tied to lignocellulosic biomass [11]. Furan derivates, which include furfural and 5-hydroxymethyl-2-furaldehyde (HMF), weak acids, mainly acetic-, formic- and levulinic acid and phenolic compounds such as vanillin.

Depending on the yeast strain and the concentration of furan derivates, HMF and furfural can decrease the volumetric ethanol yield and productivity, inhibit growth and cause a longer lag phase [12]. *In vitro* measurements of inhibitory mechanisms of HMF and furfural have shown direct inhibition of alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenease (ALDH) purified from *Saccharomyces cerevisiae* [13]. Increased levels of acetaldehyde excretion has been shown when furfural was added to the growth media [14]. This suggests that the reduction of furans by yeast results in NAD(P)H depletion [14]. Metabolic flux analysis has shown that furfural can affect the enegry metabolism in yeast by interacting with glycolytic- and TCA fluxes [15]. Also, furfural can cause vacuole-,

mitochondrial-, chromatin- and actin damage as well as the accumulation of reactive oxygen species [12].

The weak acids acetic-, formic- and levulinic acid at too high concentrations inhibit yeast fermentation by decreasing ethanol yield and biomass formation [16]. The inhibitory mechanism of weak acids is anion accumulation and uncoupling [17]. Undissociated forms of weak acids can diffuse into the cell membrane and dissociate due to increased intracellular pH which results in decreased cytosolic pH. [18–20]. Membrane-bound ATPase pumps protons out of the cell at the expense of ATP hydrolysis due to the decreased intracellular pH [19]. As a result, less ATP is used for biomass formation. Furthermore, weak acids have been shown to inhibit yeast growth by decreasing the uptake of aromatic amino acids, possibly as a result of strong inhibition of Tat2p amino acid permease [21]. However, low concentrations of acetic-, formic-, and levulinic acid have been shown to increase ethanol yield [16, 22]. Low concentrations of these acids may stimulate production of ATP under anaerobic ethanol production [16].

Phenolic compounds have been shown to decrease the biomass yield, growth rate, ethanol productivity and ethanol yield [23]. Low molecular weight phenolic compounds are generally more inhibitory than high molecular weight compounds [23]. A proposed mechanism is the action of weak acidic phenolic compounds on the electrochemical gradient by transporting protons back through the mitochondrial membrane [24].

2.2 Simultaneous saccharification and fermentation process overview

The overall process design of simultaneous saccarification and fermentation (SSF) can generally be described in a three step process (Figure 2.1) [25]. The first part of the process is the pretraetment. Here, the purpose is to maximize the yield of monomeric sugars and rate of hydrolysis [10]. Most inhibitors are produced during this process step. Prior to the pretreatment, the raw material goes through a size reduction by methods such as chipping or milling to break down the woody structure of the lignocellulosic biomass. The second part of the process is the enzymatic hydrolysis and fermentation. These steps can also be performed separately, referred to as separate hydrolysis and fermentation (SHF) [25]. Finally, the ethanol is separated from the lignin fraction, commonly by distillation [25].

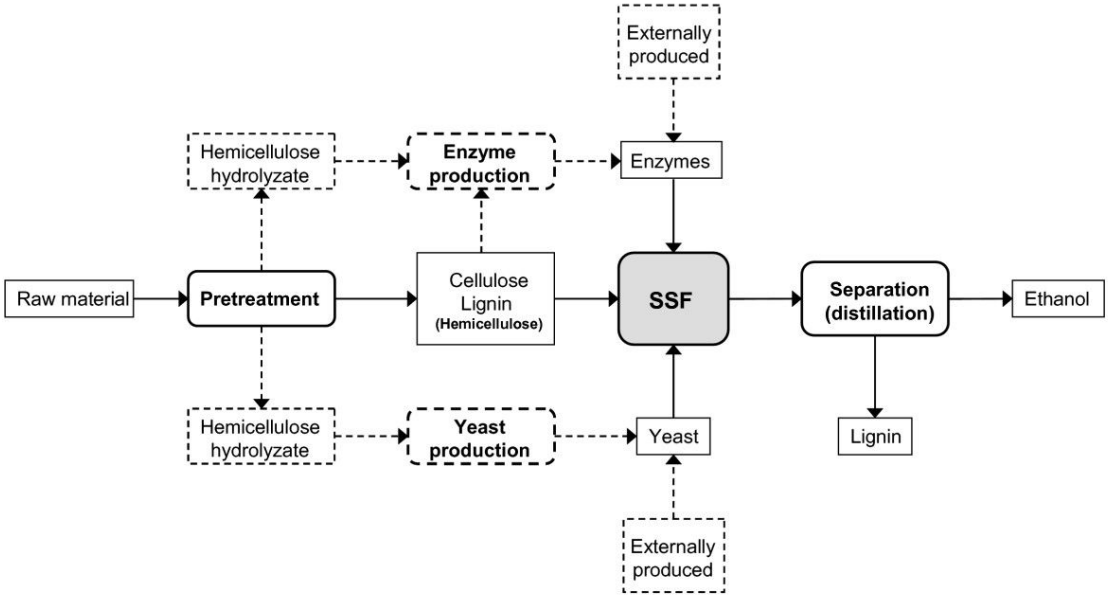


Figure 2.1: Overview of the SSF process steps [25]

2.3 Pretreatment methods

The purpose of the pretreatment is to increase the yield of monomeric sugars and the rate of hydrolysis in the subsequent process steps [10]. The pretreatment can impact other downstream processes of hydrolysis and fermentation in bioethanol production [26]. Too severe pretreatment conditions can result in high sugar degradation in the substrate. Too mild pretreatment conditions will not be effective in breaking down the wood structure, resulting in inefficient hydrolysis. There are three main types of pretreatments: physical, chemical and biological [10]. These types can be run independently or in a combination. Physical pretreatment methods mechanically break down the material by a combination of methods such as milling, grinding, chipping and uncatalyzed steam pretreatment [10]. Physical pretreatment methods aim to increase the digestibility of cellulose and the available surface area for enzymatic hydrolysis [10].

Chemical pretreatment methods include organosolvation and the chemical breakdown of the lignocellulosic material by acidic or alkaline chemicals as well as ozone and ionic liquids [27]. Alkaline pretreatment methods have been described to be more efficient on agricultural residues compared to wood residues [27]. Alkaline pretreatments are efficient for lignin solubilization, increase the cellulose digestibility and cause less glucose degradation compared to acid pretreatment methods [27]. Suitable chemicals for alkaline pretreatments are ammonium hydroxides, calcium, sodium and potassium [27]. One drawback of alkaline pretreatments is the potential loss of fermentable sugars as the hemicellulose fraction is dissolved within the lignin fraction [27].

The main reason for using acid pretreatment is its ability to solubilize the hemicellulose fraction to increase the accessibility of cellulose for the enzymes [27]. Acids at too high concentrations can cause increased operational costs due to acid recovery and equipment corrosion [27]. Furthermore, high concentration acids in pretreatment may cause the formation of compounds inhibitory to ethanol fermentation [27]. Dilute sulphuric acid is a commonly used acid while other acids such as hydrochloric acid, phosphoric acid and nitric acid also have been studied [28].

Ionic liquids consist purely of ionic species and are often fluid at room temperature [28]. They are generally composed of a salt where the cation has a low degree of symmetry and one or both ions are large, resulting in a reduced melting point [28]. Cellulosic materials recovered from ionic liquids have been found to be highly accessible to enzymatic hydrolysis by cellulases due to its porous and amorphous structure [28]. Processes that include ionic liquids have been shown to be less energy demanding, more environmentally friendly and easier to operate compared to alkaline and acid pretreatment processes [28]. However, ionic liquids are expensive.

Ozone is an oxidant that shows high delignification efficiency and avoids the formation of inhibitory compounds [27]. Ozone pretreatment is usually performed at room temperature and normal pressure [27]. However, ozonolysis is economically unfavorable due to the high costs related to ozone [27]. The main advantage of organosolvation as pretreatment is the recovery of very pure lignin as by-product compared to other chemical pretreatments [27]. Another drawback of organosolvation is the required separation prior to hydrolysis and fermentation as the solvent can be inhibitory to enzymatic hydrolysis and fermentation [27].

Acid-catalyzed steam pretreatment is a physico-chemical pretreatment method that combines physical and chemical pretreatments. Here, acids are added to the lignocellulosic biomass which then is subjected to pressurized high-temperature steam for a certain period, after which it is quickly depressurized [27]. The sudden pressure reduction causes fibers to separate due to the explosive decompression and the high temperature promotes autohydrolysis due to acetic acid formation from acetyl groups found in the hemicellulose [27]. The major factors affecting the efficiency of the acid-catalyzed steam pretreatment are temperature, residence time and particle size. The combined effect of temperature and time can be described by the the so-called severity factor [27]. The major advantages of this pretreatment method are possibilities for significantly lower environmental impact, low capital investments, less hazardous process chemicals and conditions, potential for high energy efficiency and complete sugar recovery compared to other pretreatment methods [27]. This method is one of the older and more effective pretreatments, however, a drawback that has been observed when using woody substrates is a reduced delignification which results in lower enzymatic hydrolysis of the cellulose [29].

Biological pretreatment methods utilize brown, white or soft rot fungi to degrade lignocellulosic material [10]. Biological pretreatments are in general safe and energy saving [10]. However, hydrolysis rates are lower compared to other pretreatments

although being considered more environmentally friendly [10]. The approach does not require expensive equipment, addition of chemical agents or additional energy to remove the lignin [30]. Drawbacks are the slow processing of delignification and the carbohydrate loss in the substrate as the microorganisms consume parts of cellulose and hemicellulose during pretreatment, leaving less sugars and polysaccharides left for enzymatic hydrolysis and fermentation [30].

2.4 Enzymatic hydrolysis

The hydrolysis of cellulose involves three major classes of cellulase enzymes, cellobiohydrolases, endo-β-1,4-glucanases and β-glucosidases [31]. Cellobiohydrolases act by cleaving the ends of the cellulose polymers, endo-β-1,4-glucanases act by internally cleaving the cellulose polymers and β-glucosidases hydrolyze short chain glucooligosaccharides to glucose [32]. The xylose parts of hemicellulose can also be hydrolyzed into xylose by xylanases [33]. Cellulose that are a part of lignin complexes are more difficult to hydrolyze. Other than an effective pretreatment, lignin degradation can be enzymatically assisted by lignin peroxidase, laccase enzymes and manganase dependent peroxidase by acting on the phenolic parts of the lignin [33]. Lytic polysaccharide monooxygenases (LPMOs) are also able to cleave polysaccharide links [34]. LPMOs can act on crystalline cellulose as well as on hemicellulose by direct oxidative attack on the polymer chains via a flat active site that has a centrally located copper ion [34].

2.5 Simultaneous saccharification and co-fermentation

After the raw material has been pretreated and hydrolyzed, it is fermented. One operating option is to perform the enzymatic hydrolysis and fermentation together, called SSF [25]. Separation and refinement of the ethanol from the fermented slurry is one of the major energy consuming parts of the process, up to 80% in some cases [35]. Economically beneficial and efficient bioethanol production thus requires high ethanol yields and titers. It is possible to reduce the distillation cost by operating at high water-insoluble solid (WIS) contents [36]. Operating at high WIS content does present challenges, such as increased viscosity, resulting in a reduced efficiency of heat and mass transfer as well as increased power consumption due to higher demands on mixing [36]. Furthermore, higher substrate content will lead to increased amounts of inhibitors which can have a negative effect on the final titer, productivity and yield [36]. Increasing the enzyme dosage during high-WIS SSF could reduce viscosity. However, the process economy and environmental impact is considerably affected by enzyme usage [36, 37]. Operating in fed-batch mode can help deal with these challenges by allowing high enzyme to substrate ratios through the process by continuously feeding high WIS substrate, yeast and enzymes [38, 39].

Enzyme feeding has been shown to maintain low glucose concentration, favouring higher xylose uptake in yeast [40]. Furthermore, substrate feeding has been shown to reduce viscosity, increasing total amount of WIS that can be added in the process [40]. It is also possible to increase the cell tolerance to lignocellulosic inhibitors by pre-adapting yeast to the lignocellulosic media in propagation cultures [41, 42]. One advantage of SSF compared to SHF is reduced end-product inhibition of hydrolysis by cellobiose and glucose due to the rapid conversion of the sugars [25, 43]. Furthermore, the SSF allows no separation of glucose from the lignin fraction after separate enzymatic hydrolysis. This bypasses a potential sugar loss [25]. Due to the reduction of required vessels, capital investments are reduced [25]. Tied to the lower equipment costs, the operation is easier since less equipment is needed [43]. The main disadvantage of SSF is the different temperature optima for enzymatic hydrolysis and fermentation [25, 43]. However, the utilization of thermophilic strains can solve this drawback. In SHF processes, enzymatic hydrolysis and fermentation can be optimized independently [25]. Further more, in SSF, yeast and enzymes cannot be reused because their separation from the lignin after the fermentation is currently too difficult [25].

The pretreatment and enzymatic hydrolysis of the lignocellulosic biomass typically release not only hexose sugars such as glucose but also pentose sugars such as xylose and arabinose together with inhibitors [44]. Simultaneous saccharification and co-fermentation (SSCF) is a mode of operation that utilizes recombinant yeast strains that can simultaneously ferment glucose and xylose [44].

2.6 Yeast xylose fermentation

The yeast *S. cerevisiae* has been used for production of food and beverages throughout the ages. Over the last decades, it has been the most industrially utilized production microorganism in biofuel production using fermentation of hexose sugars for its robustness, high ethanol production and high ethanol tolerance [45]. However, wild-type yeast are unable to utilize xylose, which is a major component of lignocellulosic biomass [46]. *S. cerevisiae* can utilize the same transport system for xylose uptake as for glucose uptake. However, K_M values for the xylose transport are reported to be at least 5-200 fold higher compared to glucose transport [47]. This means that glucose and xylose are only consumed simultaneously at glucose limiting conditions [48].

There are two main different xylose utilizing pathways that have been implemented into *S. cerevisiae* from other xylose utilizing microorganisms such as *Scheffersomyces stipitis* [49]. One of the pathways is the xylose isomerase (XI) pathway, which catalyzes the isomerisation of D-xylose to to D-xylulose [49]. This pathway is most common in xylose utilizing bacteria [50]. This pathway has previously been integrated into *S. cerevisiae* for ethanol production [51]. However, this pathway is not commonly used for fungi. The other pathway is the xylose reductase (XR)/xylitol

dehydrogenase (XDH) pathway, which catalyzes a two step reaction where D-xylose is reduced to D-xylitol and D-xylitol is then oxidized to D-xylulose which is dependent on the co-factors NAD(P)H and NAD^+ respectively [49]. *S. cerevisiae* can slowly metabolize xylulose, the ketoisomer of xylose [46]. It is then possible to enable xylose consumption in *S. cerevisiae* by introducing a xylose isomerisation pathway from another xylose utilizing microorganism [46].This pathway is most common in most xylose utilizing fungi [50]. The *S. cerevisiae* strain KE6-12A, used in this project, utilizes the XR/XDH pathway [52]. In either pathway, D-xylulose is then phosphorylated into D-xylulose 5-phosphate by xylulokinase (XK), which is further metabolized in the pentose phosphate pathway [50]. This reaction is dependent on the phosphorylation potential and energy charge of the cell as it uses ATP. There is an intracellular redox imbalance that occurs in most naturally xylose fermenting yeasts due to the difference in co-enzyme specificities of XR and XDH [50]. XR is primarily NADPH dependent while XDH is strictly NAD^+ dependent [50]. This results in excretion of xylitol as by-product [50].

3

Methods

3.1 Raw material

The pre-treated raw material was provided by the SP Biorefinery Demo Plant (Örnsköldsvik, Sweden). The spruce tips, needles and branches were pre-treated by H_2SO_4 (1% (w/v)) catalyzed steam treatment where the temperature and residence time in the pretreatment reactor were varied into 13 different combinations according to a design of experiments. These 13 different combinations are divided into 3 groups for further reference. The star points (materials MAT.IB.100-102 and MAT.IB.112), the center points (MAT.IB.106-109) and the edge points (MAT.IB.103-105 and MAT.IB.110-111). The compositions and pretreatment conditions of all 13 materials have previously been determined (Table A.1) [53].

3.2 Strain and media

The strain used in all experiments was the recombinant *S. cerevisiae* mutant KE6-12A [54]. It was chosen for it's ability to co-ferment glucose and xylose. The strain was maintained as a frozen 30 % glycerol stock in -80 °C. The media used in all shake flask preculture cultivations was YPD medium with yeast extract (10 g l^{-1}), bacto peptone (20 g l^{-1}) and glucose (20 g l^{-1}). The media used in the bioreactor preculture cultivations was YPD medium with yeast extract (10 g l^{-1}), bacto peptone (20 g l^{-1}), glucose (10 g l^{-1}) and the addition of xylose (5 g l^{-1}) to adapt the cells for xylose consumption. The pH of the bioractor YPD medum was also initially adjusted to pH 5.0 with HCl (1 M). YPD agar plates with yeast extract (10 g l^{-1}), bacto peptone (20 g l^{-1}), glucose (20 g l^{-1}) and agar (20 g l^{-1}) were used to measure colony forming units.

3.3 Preculture fermentations

The yeast strain was propagated in pre-cultures prior to SSCF with the pre-treated spruce tips, needles and branches. The yeast was first inculated into shake flasks directly from glycerol stocks. As the cells reached stationary phase, they were transferred to a bioreactor for further propagation. This was necessary to achieve the high cell concentration needed for the fermentation in the lignocellulosic material.

3.3.1 First preculture: Shake flasks

Duplicate 500 ml shake flasks with a working volume of 100 ml, were inoculated with 400 µl glycerol stock of the yeast strain. The shake flasks were incubated in a shaking incubator (30 °C, 180 rpm). Cells from one of the duplicates were chosen as inoculum for the bioreactor.

3.3.2 Second preculture: Bioreactors

Cell suspension from the first pre-culture shake flasks was inoculated in a 3.2 l bioreactor (Labfors, Infors AG, Switzerland) with a working volume of 1 l. The bioreactor was run at 600 rpm stirring speed, 1 vvm air flow rate and pH set point of 5.0. KOH (4 M) was used as base titrant to perform one-sided pH control. As the cells reached stationary phase, 2 h after the consumption of acids, indicated by a sharp peak in pH and offgas CO_2, the cells were transferred to the SSCF shake flask system. 10 min prior to cell harvest, the stir speed was reduced to 300 rpm to adapt the cells to the lower oxygen concentrations of the SSCF shake flask system. The harvested cells were washed with sodium citrate buffer (1 M) before transfer. The cell suspension from the bioreactor was centrifuged (5 min, 5000 rpm, 4 °C). The supernatant was discarded and the pellet was re-suspended in 40 ml sodium citrate buffer (1 M). The new cell suspension was centrifuged again (5 min, 5000 rpm, 4 °C) and the supernatant was discarded. The wet cell weight (WCW) after centrifugation was recorded and resuspended in the same volume in ml of sodium citrate buffer (1 M) as the WCW recorded.

3.4 Simultaneous saccharification and co-fermentation

Each raw material was fermented in triplicate in a previously developed shake flask system [53]. The SSCF shake flask system utilized 500 ml shake flasks with a total working weight of 125 g, 10 mass % WIS, 9.5 filter paper units (FPU) (g WIS)$^{-1}$, 0.1 mg cells (g WIS)$^{-1}$ and 50 mmol kg^{-1} sodium citrate buffer. The enzymes used

for the hydrolysis was the Cellic CTEC 2 mixture (LOT #: SLBS6227). The shake flasks were incubated in a shaking incubator (31.5 °C, 180 rpm). The environment inside the shake flasks was kept micro aerobic by nitrogen sparging. The nitrogen gas was moisturized by with sterile MilliQ water. The initial pH of the fermented slurry was adjusted to 5.0 with NaOH (4 M). The fermentation was run for 72 h and samples were taken at 0, 3, 6, 9, 24, 33, 48, 57 and 72 h. Samples were taken by opening the shake flasks and transferring complete slurry to falcon tubes via custom cut serologigal pipette tips. The liquid and solid fraction of the samples were separated by centrifugation (5000 rpm, 12 min, 4 °C).

3.5 Analytical methods

Samples taken during SSCF experiments were analyzed in terms of cell viability, contents of the liquid fraction and WIS.

3.5.1 Cell viability

Cell viability was evaluated using colony-forming unit (CFU) assays and cell enumeration using methylene blue staining in a hemocytomceter (Neubauer chamber). Viable cells can be defined as alive, metabolically active and culturable cells.

Samples taken at 0, 24, 48, and 72 h were diluted to a 10^{-4} dilution and streaked on YPD agar plates. For materials MAT.IB.100 to MAT.IB.102 as well as one of the triplicates of MAT.IB.103, the samples were diluted to a 10^{-1} dilution before streaking for all time points except 72 h. Each shake flask sample was streaked in triplicate, generating triplicate plates per shake flask triplicate. The plates were incubated at 30 °C for three days. The number of CFUs were then calculated.

The colony forming units (CFU, CFU ml^{-1}) where calculated by multiplying the number of colonies counted (n) with the dilution factor (d) and divided by the volume (V) used to inoculate the plates (0.1 ml) (Equation 3.1).

$$CFU = \frac{nd}{V} \tag{3.1}$$

Samples taken at 0, 3, 6, 24, 33, 48, 57 and 72 h were serial diluted to a 10^{-1} dilution and the number of cells was counted in a Neubauer chamber. 20 µl of methylene blue was added per ml of sample to differentiate dead and alive cells. For materials MAT.IB.100 to MAT.IB.102 as well as one of the triplicates of MAT.IB.103, the samples were diluted to a 10^{-4} dilution before cell counting for all time points except 72 h.

The cell concentration (X, cells ml^{-1}) was calculated by multiplying the number of alive cells (N) with the volume of the square (V) divided by the dilution factor (d) and the number of squares counted. Five squares were counted, the four edge squares and the center square (Equation 3.2).

$$X = \frac{NV}{5d} \tag{3.2}$$

The specific growth rate (μ, h^{-1}) was calculated as an average between consecutive time points using the previously calculated cell concentration (X) (Equations 3.3-3.7).

$$\frac{dX}{dt} = \mu X \tag{3.3}$$

$$\frac{1}{X} dX = \mu dt \tag{3.4}$$

$$\int_{X_2}^{X_1} \frac{1}{X} dX = \mu \int_{t_2}^{t_1} dt \tag{3.5}$$

$$ln(\frac{X_1}{X_2}) = \mu(t_1 - t_2) \tag{3.6}$$

$$\mu = \frac{ln(\frac{X_1}{X_2})}{t_1 - t_2} \tag{3.7}$$

3.5.2 Fermentation products and sugars

Samples from all time points were centrifuged (5000 rpm, 12 min, 4 °C). The liquid fraction was removed and filtered through nylon filters (0.2 µm). The liquid fraction samples were then analyzed via high-performance liquid chromatography (HPLC) [55]. A Jasco UV-RI HPLC system using a Phenomenex Rezex ROA-Organic Acid H+ (8 %) 150 x 7.8 mm LC column. Both a RI-4030 RI and a UV-4075 UV/VIS detector were used where the detection was made at 210 nm. The samples were run for 45 min and eluted at 80 °C using H_2SO_4 (5mM) as mobile phase at a flow rate of 0.8 ml min^{-1}. Standards used were serial diluted into 1:1, 1:2, 1:4, 1:8, 1:32 and 1:64 times dilutions (Table B.1).

The overall ethanol yield on glucose was calculated by dividing the titer of ethanol at a specific time point ($C_{\text{Ethanol}}(t)$) with the titer of glucose at time 0 ($C_{\text{Glucose}}(t_0)$). The total liquid volume (V_{L}) in the shake flasks was used to achieve a unit of g_{Ethanol} per g_{Glucose}. The total working weight of the shake flask (m_{tot}) in grams adjusted for the fraction of WIS (WIS) together with the fraction of cellulose ($F_{\text{Cellulose}}$) and the anhydrous correction factor for cellulose (0.90) was used to take the amount of cellulose into account (Equation 3.8).

$$Y_{\text{Ethanol/Glucose}} = \frac{C_{\text{Ethanol}}(t) V_{\text{L}}}{C_{\text{Glucose}}(t_0) V_{\text{L}} + 0.90 F_{\text{Cellulose}} WIS m_{\text{tot}}} \tag{3.8}$$

The overall ethanol yield on glucose and xylose was calculated similarly to Equation 3.8 but with the addition of xylose concentration at time 0 ($C_{\mathrm{Xylose,t_0}}$), the fraction of xylan (F_{Xylan}) and the anhydrous correction factor for xylan (0.88) (Equation 3.9)

$$Y_{\mathrm{Ethanol/Glucose+Xylose}} = \frac{C_{\mathrm{Ethanol}}(t)V_{\mathrm{L}}}{C_{\mathrm{Glucose}}(t_0)V_{\mathrm{L}}+0.90F_{\mathrm{Cellulose}}WISm_{\mathrm{tot}}+C_{\mathrm{Xylose}}(t_0)V_{\mathrm{L}}+0.88F_{\mathrm{Xylan}}WISm_{\mathrm{tot}}} \tag{3.9}$$

The volumetric ethanol production rate ($r_{\mathrm{Ethanol}}(t)$) at a specific time point was calculated by dividing the titer of ethanol of the two surrounding time points with the time of the specific time point. The rate is also adjusted based on the uneven distance between the time points (Equation 3.10).

$$r_{\mathrm{Ethanol}}(t_{\mathrm{x}}) = \frac{t_{\mathrm{x}}-t_{\mathrm{x\text{-}1}}}{t_{\mathrm{x+1}}-t_{\mathrm{x\text{-}1}}}\frac{C_{\mathrm{Ethanol}}(t_{\mathrm{x+1}})-C_{\mathrm{Ethanol}}(t_{\mathrm{x}})}{t_{\mathrm{x+1}}-t_{\mathrm{x}}} + \frac{t_{\mathrm{x+1}}-t_{\mathrm{x}}}{t_{\mathrm{x+1}}-t_{\mathrm{x\text{-}1}}}\frac{C_{\mathrm{Ethanol}}(t_{\mathrm{x}})-C_{\mathrm{Ethanol}}(t_{\mathrm{x\text{-}1}})}{t_{\mathrm{x}}-t_{\mathrm{t_{x\text{-}1}}}} \tag{3.10}$$

3.5.3 Water insoluble solids

The solid fraction of the centrifuged samples were analyzed in terms of WIS content [56]. The solid fraction samples were centrifuged (20 min, 5000 rpm, 4 °C). The glucose concentration was measured in the supernatant using colorimetric test strips (MQuant). If the glucose concentration was greater than 50 mg l^{-1}, the supernatant was discarded and the pellet was resuspended in 30 ml MilliQ water. The centrifugation and resuspending was repeated until the glucose concentration was lower than 50 mg l^{-1}.

Aluminum dishes were predried in a convection oven at 105 °C for 24 h and weighed. 1-3 g of wet solid fraction samples were placed on the pre-dried aluminum dishes inside a convection oven at 105 °C for 24 h. The dried samples were then placed inside a desiccator and let to cool down for 30 min and then weighed.

The WIS (WIS, g dry sample weight / g wet sample weight) in the samples was calculated as a mass fraction using the wet- and dry weights of the solid fraction samples. The wet samples were washed to reduce glucose concentration to below 50 mg l^{-1} and taken into account in the calculations, ($m_{\text{Sample weight after washing}}$) and ($m_{\text{Sample weight before washing}}$) (Equation 3.11).

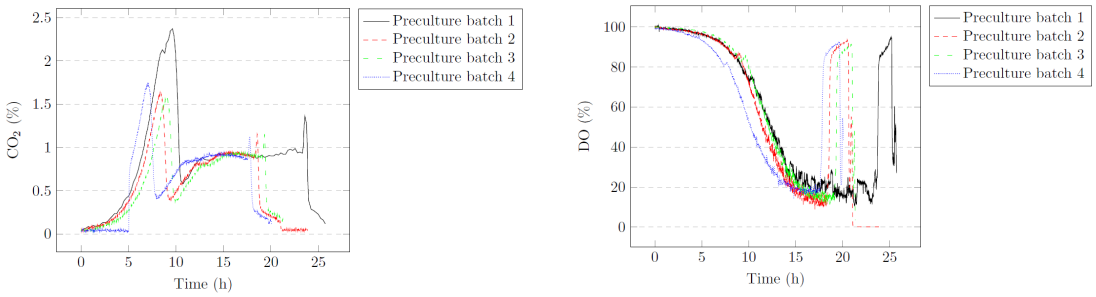
$$WIS = 100\frac{m_{\text{Dry sample weight + Aluminum dish}} - m_{\text{Aluminum dish}}}{m_{\text{Wet sample weight}}}\frac{m_{\text{Sample weight after washing}}}{m_{\text{Sample weight before washing}}} \tag{3.11}$$

4

Results

4.1 Preculture fermentations

The CO_2 and the dissolved oxygen (DO) in the offgas was used to predict the state of the cells during the fermentation (Figure 4.1). There were 2 main peaks in the CO_2 offgas. The first peak was the end of the sugar consumption phase and the second peak was the end of the ethanol consumption phase indicated by rapid consumption of accumulated acetate [57]. Cells were harvested 10 min after the second CO_2 offgas peak as it was assumed that the cells were in stationary phase at this point and thus more robust [58].



(a) **(b)**

Figure 4.1: Offline CO_2 and DO analysis of the preculture batch fermentations. (a) CO_2 percentage in the offgas sensor. (b) DO percentage in the offgas sensor.

4.2 Fermentation products and sugars

Values below the limit of detection are presented as 0. These concentrations correspond to a peak area lower than a 1:64 dilution of the standard used to identify the peak. Ethanol was produced in titers up to about 12 gL^{-1} during the first 9 h of SSCF (Figure 4.2, Table C.1). After the first 9 h, the ethanol production leveled

out and decreased. This was true for all points in the design of experiments with the exeption of material MAT.IB.102. The star points showed slightly higher titers compared to the other points (Figure 4.2a).

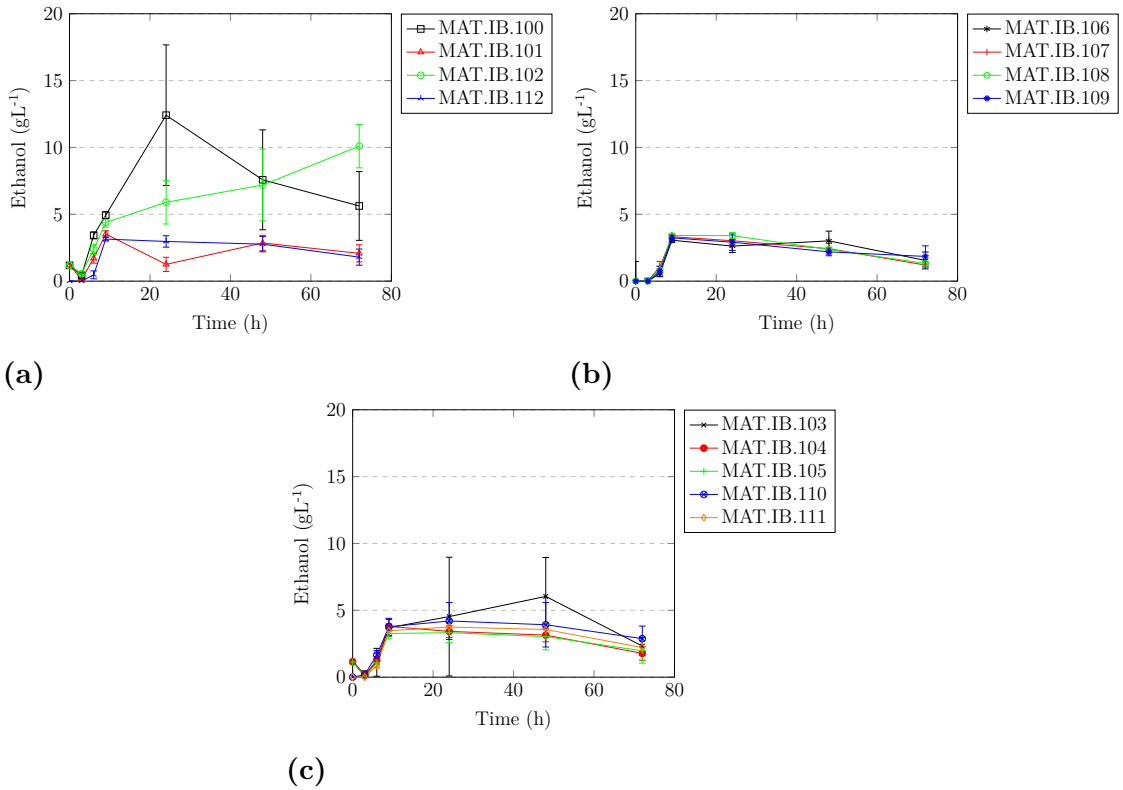


Figure 4.2: Ethanol concentration with standard deviation for all 13 materials. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

Glucose was initially released during the first 3 h after which it was rapidly consumed for 6 h (Figure 4.3, Table C.1). After the first 9 h of SSCF the glucose concentration increased in all materials with the exceptions of MAT.IB.100, MAT.IB.102, MAT.IB.103 and MAT.IB.110. Glucose was steadily released rather than consumed in the centerpoints.

Hemicellulose concentrations, as the sum of xylose, mannose and galactose, were mostly steady. The hemicellulose consumption that could be observed was slower and occurred later in comparison to glucose (Figure 4.4, Table C.1). Materials MAT.IB.100, MAT.IB.102 and MAT.IB.103 are the only materials where there was noticeable hemicellulose consumption. This consumption started after 20 h of SSCF. The hydrolysis of these sugars was slower compared to the hydrolysis of glucose.

Xylitol was rapidly produced after 6 h of SSCF after which the titers leveled out (Figure 4.5, Table C.1). The concentrations were fairly similar in all materials.

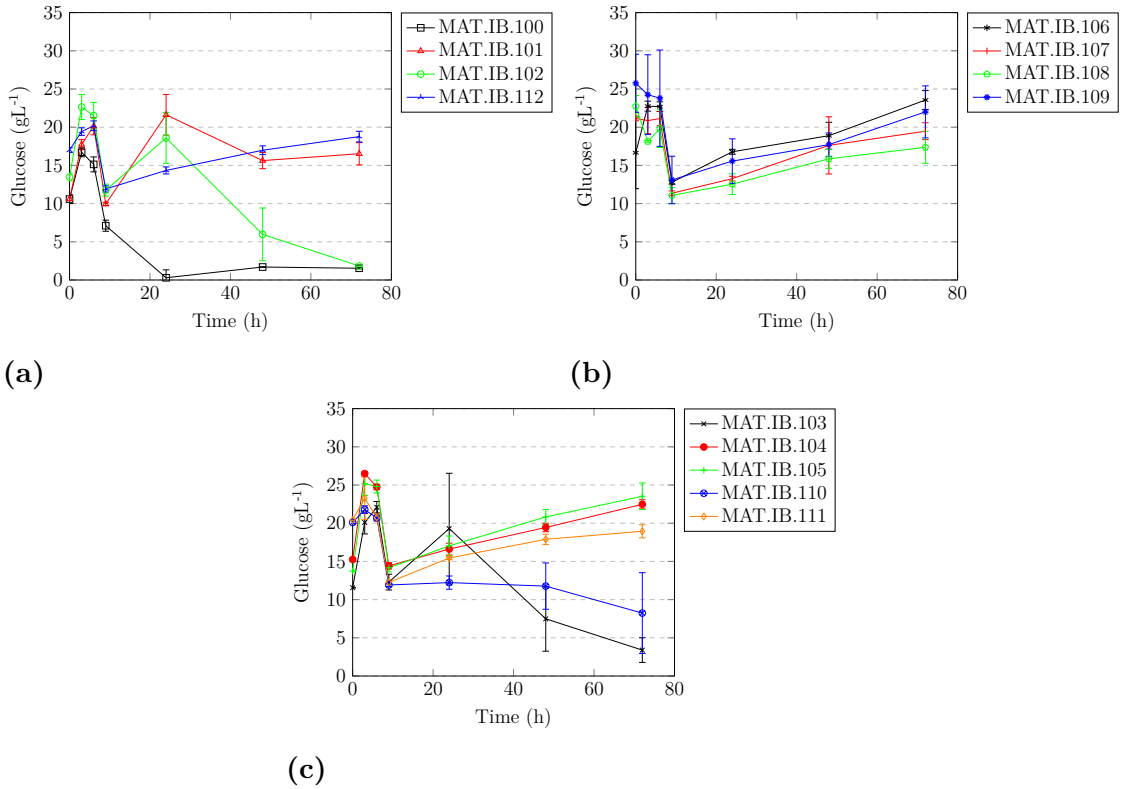


Figure 4.3: Glucose concentration with standard deviation for all 13 materials. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

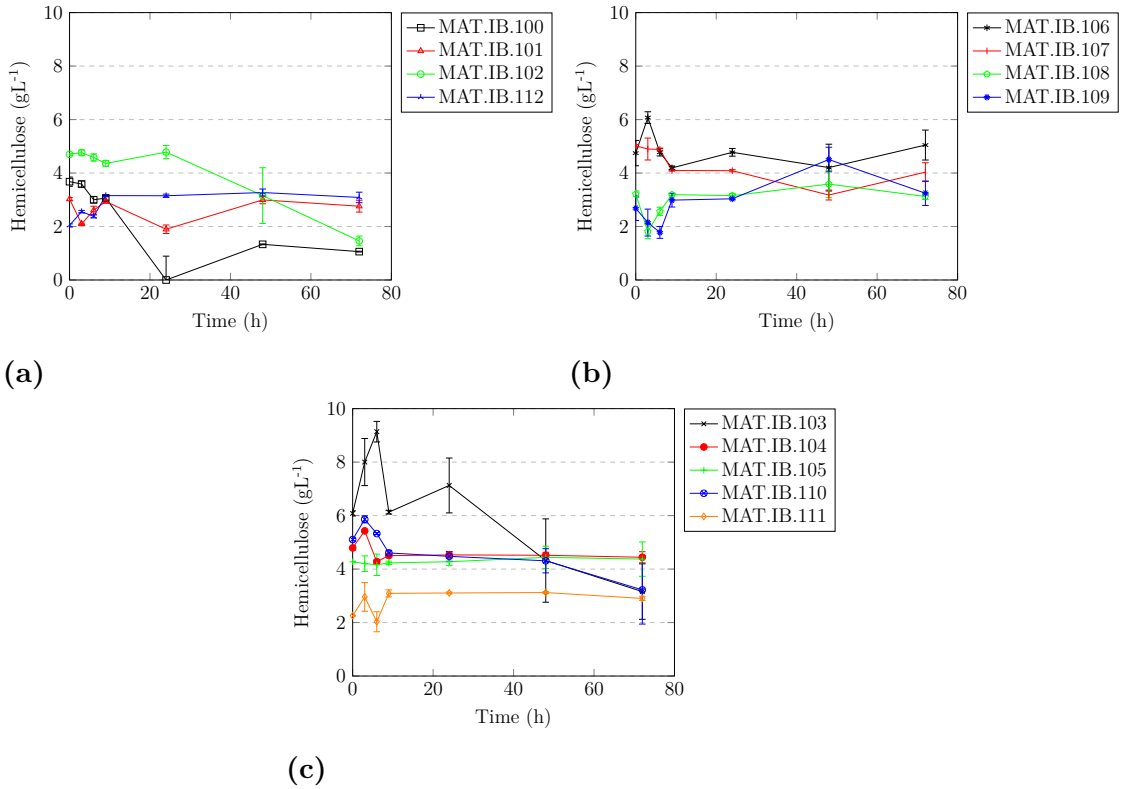


Figure 4.4: Hemicellulose concentration with standard deviation for all 13 materials. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

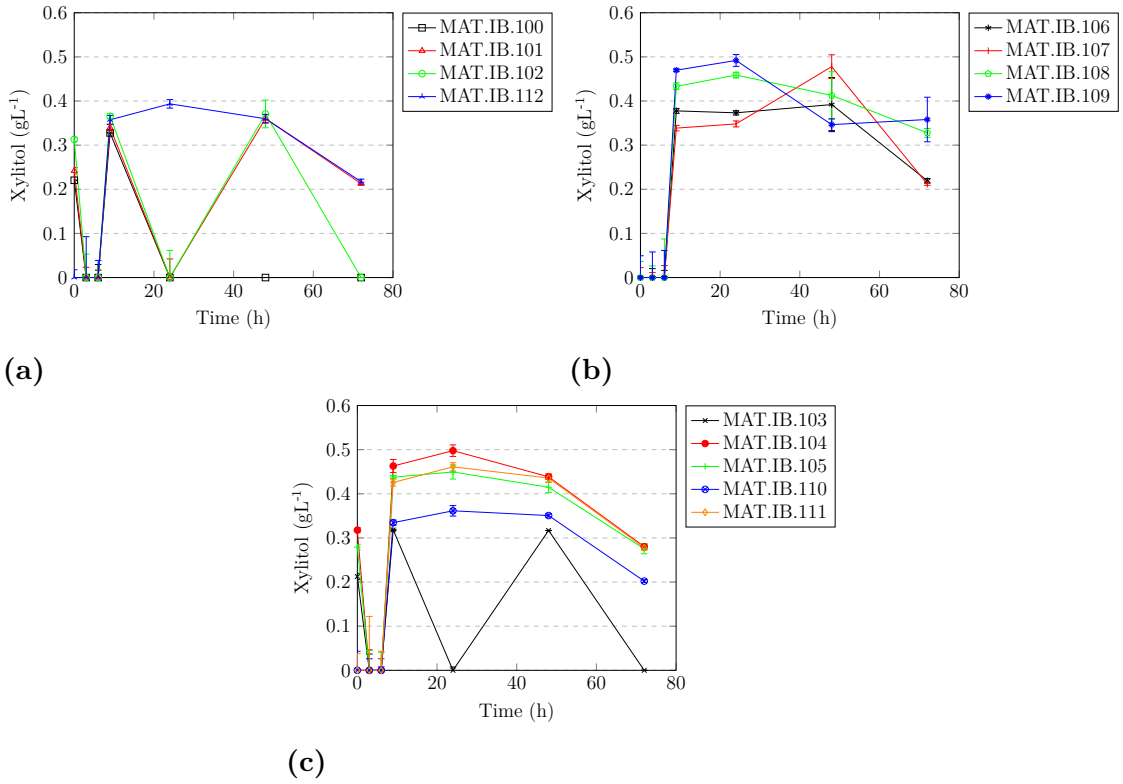


Figure 4.5: Xylitol concentration with standard deviation for all 13 materials. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

Cellobiose was initially released during the first 6 h of SSCF after which it was hydrolyzed until 24 h into the SSCF (Figure 4.6, Table C.1). After 24 h of SSCF, there was a clear trend that cellobiose was released rather than hydrolyzed.

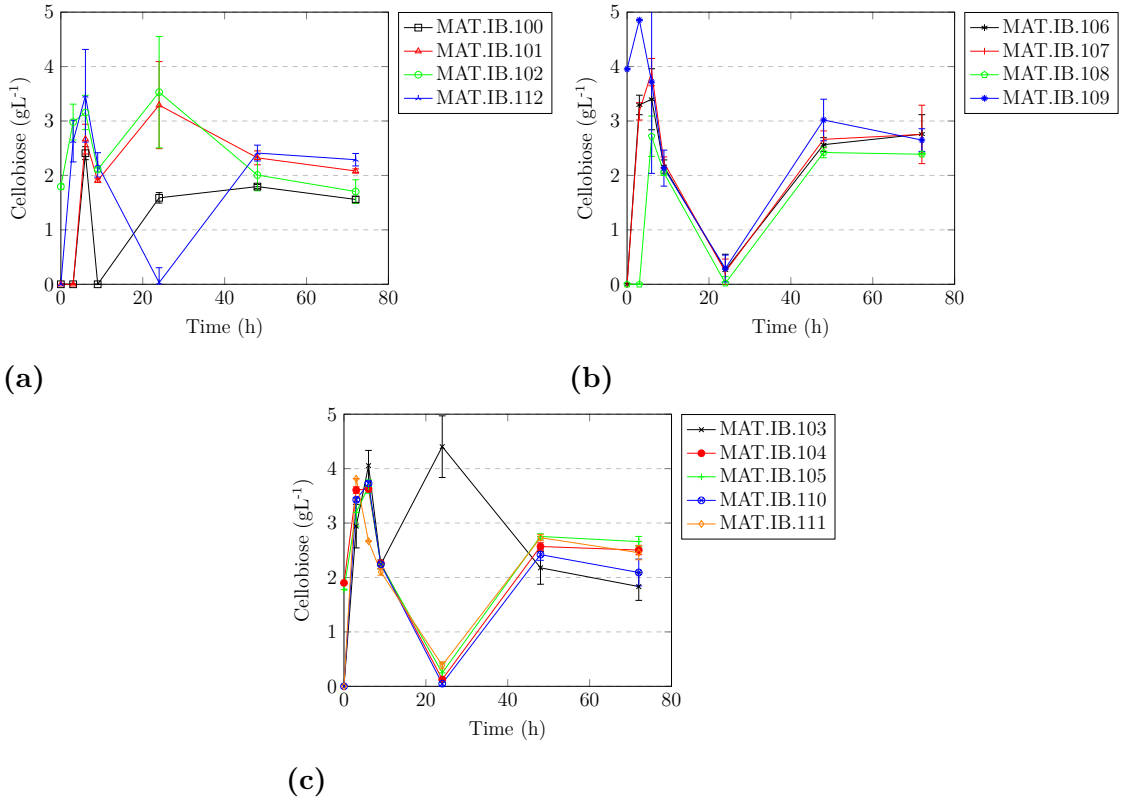


Figure 4.6: Cellobiose concentration with standard deviation for all 13 materials. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

There was an unknown compound found in all samples during HPLC analysis. It was not identified and increased in detector response during the first 24 h of SSCF after which it leveled out (Figure 4.7, Table C.1). The unknown peak co-eluted with cellobiose at 6 minutes and was detected by the UV detector.

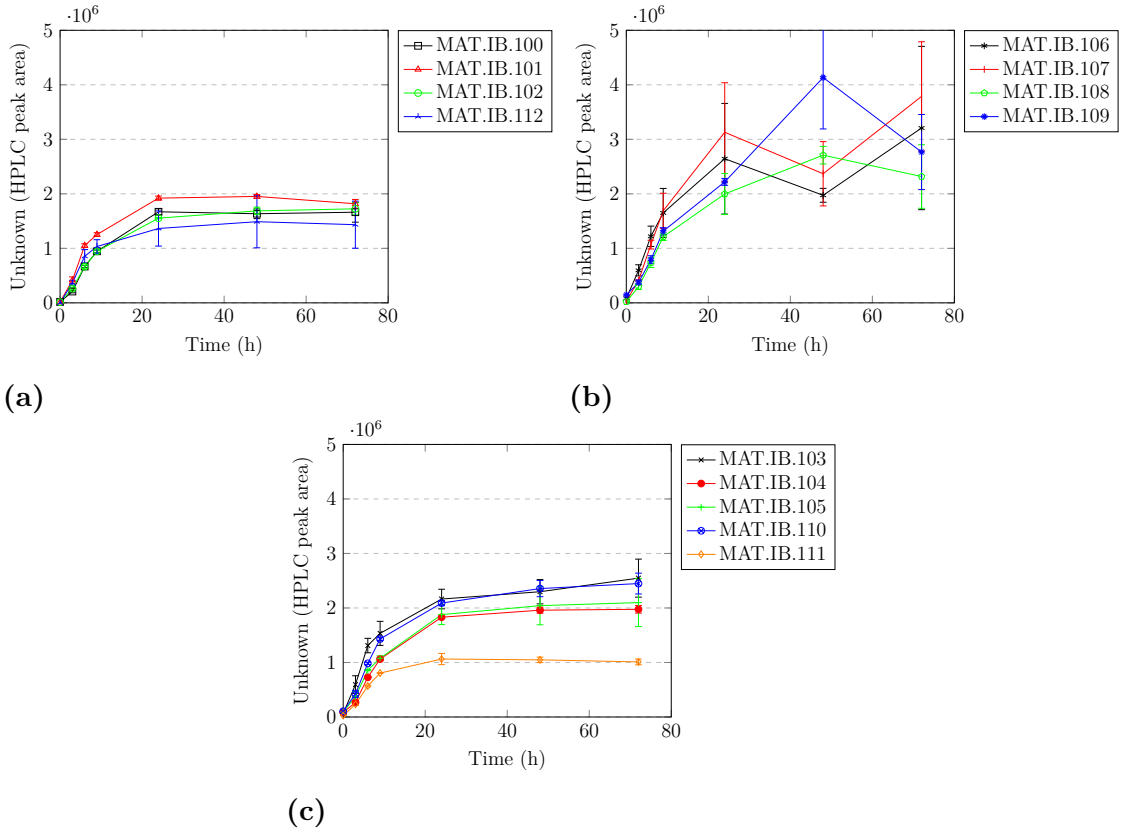


Figure 4.7: HPLC peak area with standard deviation for all 13 materials. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

4.3 Ethanol yields and rates

The final ethanol yields on both glucose and glucose together with hemicellulsoses after 72 h of SSCF are expressed as grams of ethanol per grams of sugars (Figure 4.8, Table C.2). The highest yields were observed in materials MAT.IB.100 and MAT.IB.102.

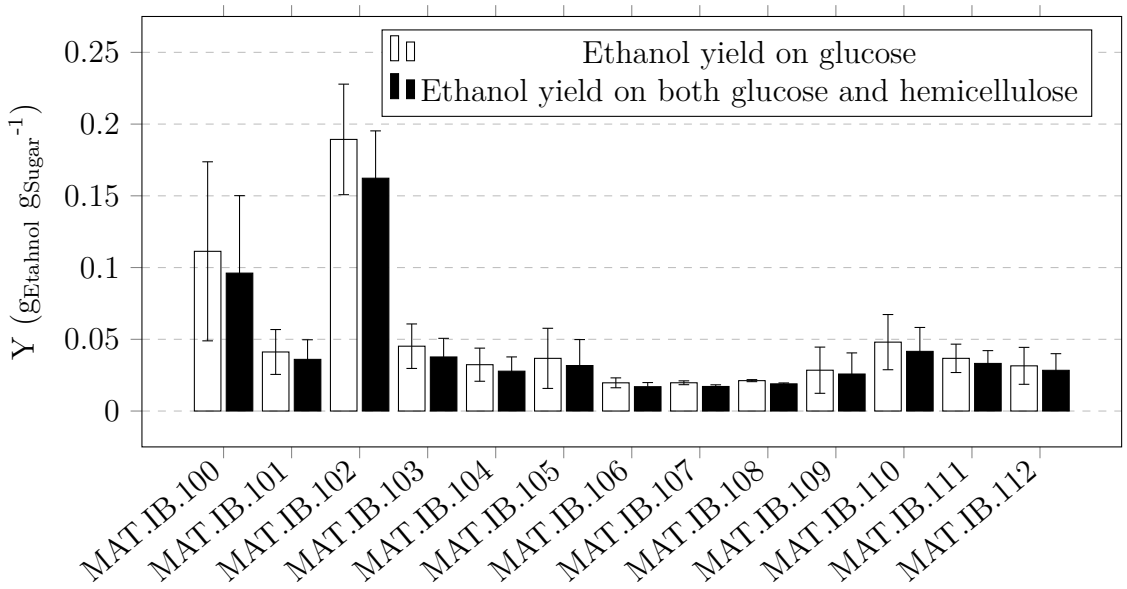


Figure 4.8: The ethanol yield of ethanol on both glucose and glucose together with hemicellulose with standard deviation after 72 h of SSCF.

The volumetric ethanol production rate fluctuated greatly over the fermentation time (Figure 4.9, Table C.2). The rates generally increased in all materials during the first 9 h of fermentation. The rates in all 13 materials, other than MAT.IB.100 and 102, were low 24 h of fermentation.

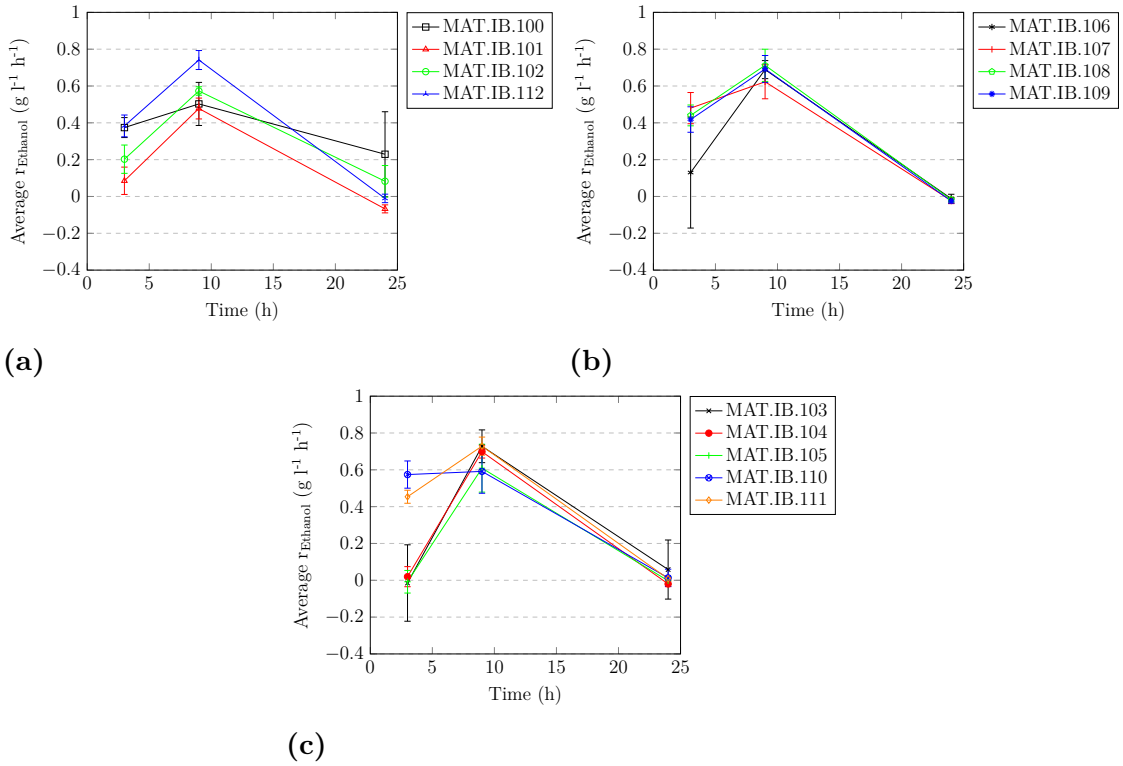


Figure 4.9: Average ethanol production rate with standard deviation for all 13 materials at 3, 9 and 24 h. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

4.4 Water insoluble solids

The WIS of all 13 materials did not show a clear trend but generally increased from an average of 9.65 % to an average of 10.6 % during 72 h of SSCF. (Figure 4.10, Table D.1).

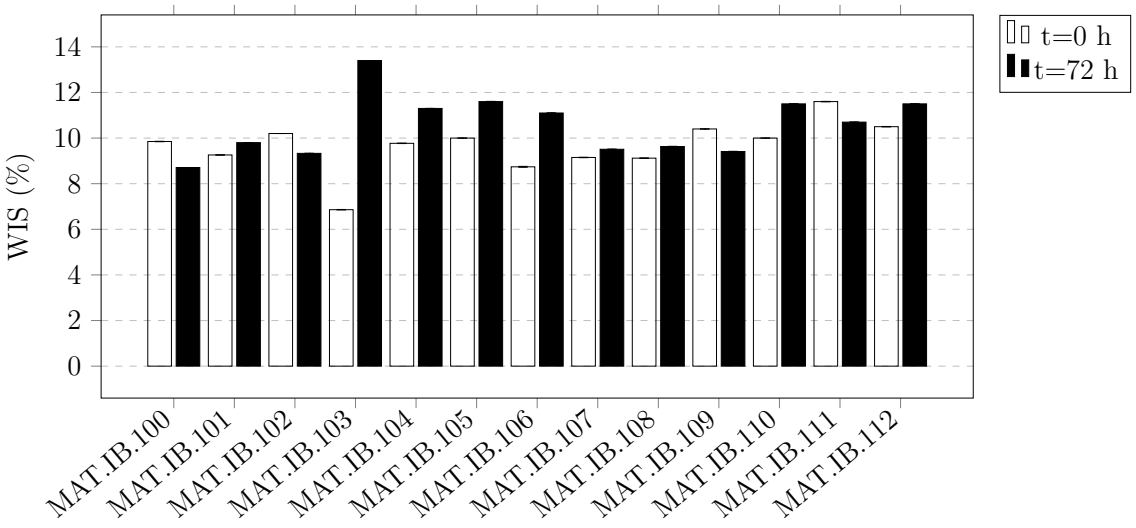


Figure 4.10: The WIS expressed as total percent of water insoluble solids for all 13 materials with standard deviation.

4.5 Cell viability

The cell viability was evaluated by counting cells in a Neubauer chamber as well as calculating CFUs. The number of cells counted in the Neubauer chamber generally decreased between 0 h and 3 h after which the cell count was overall stable (Figure 4.11, Table E.1). In the case of materials MAT.IB.100 to one of the triplicates of MAT.IB.103, the samples were diluted so much that there were no observable cells to count. Materials MAT.IB.100 to one of the triplicates of MAT.IB.103 were serially diluted to a 10^{-4} dilution while all other materials were diluted to a 10^{-1} dilution. The standard deviation of each sample was quite high.

In general, the CFU of all materials decreased after 24 h after which stable low values were reached (Figure 4.12, Table E.1). In some cases, such as MAT.IB.100 from zero to 48 h, there were too many colonies to count and it was assumed there were 500 colonies or more. 500 colonies results in a CFU of 5×10^7 CFU ml^{-1}. Materials MAT.IB.100 to one of the triplicates of MAT.IB.103 were diluted to a 10^{-1} dilution while all other materials were serially diluted to a 10^{-4} dilution.

The growth rate was calculated using Equation 3.7 and was overall low (Figure

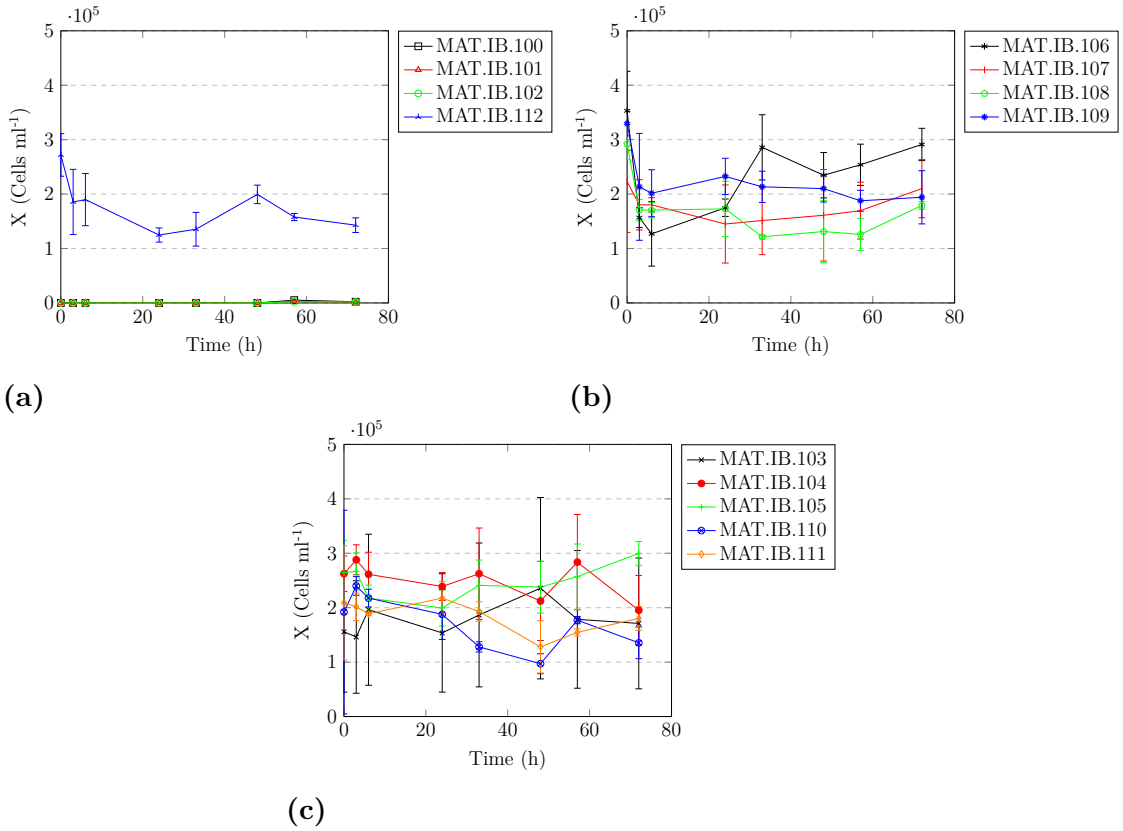


Figure 4.11: Cell concentration with standard deviation for all 13 materials measured by cell counting. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

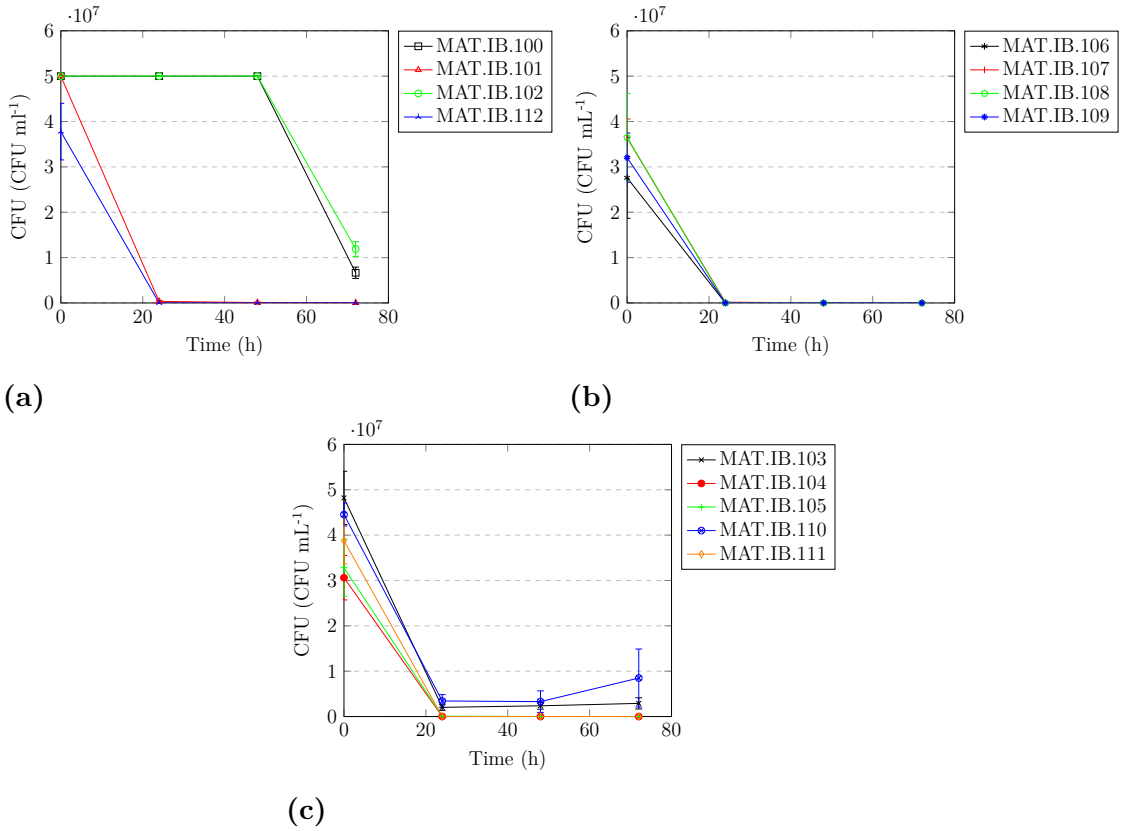


Figure 4.12: CFU with standard deviation for all 13 materials. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

4.13, Table E.1). Materials MAT.IB.100, MAT.IB.101 and MAT.IB.102 showed the highest growth rates between 0.2 and 0.4 h^{-1} at 57 h of SSCF.

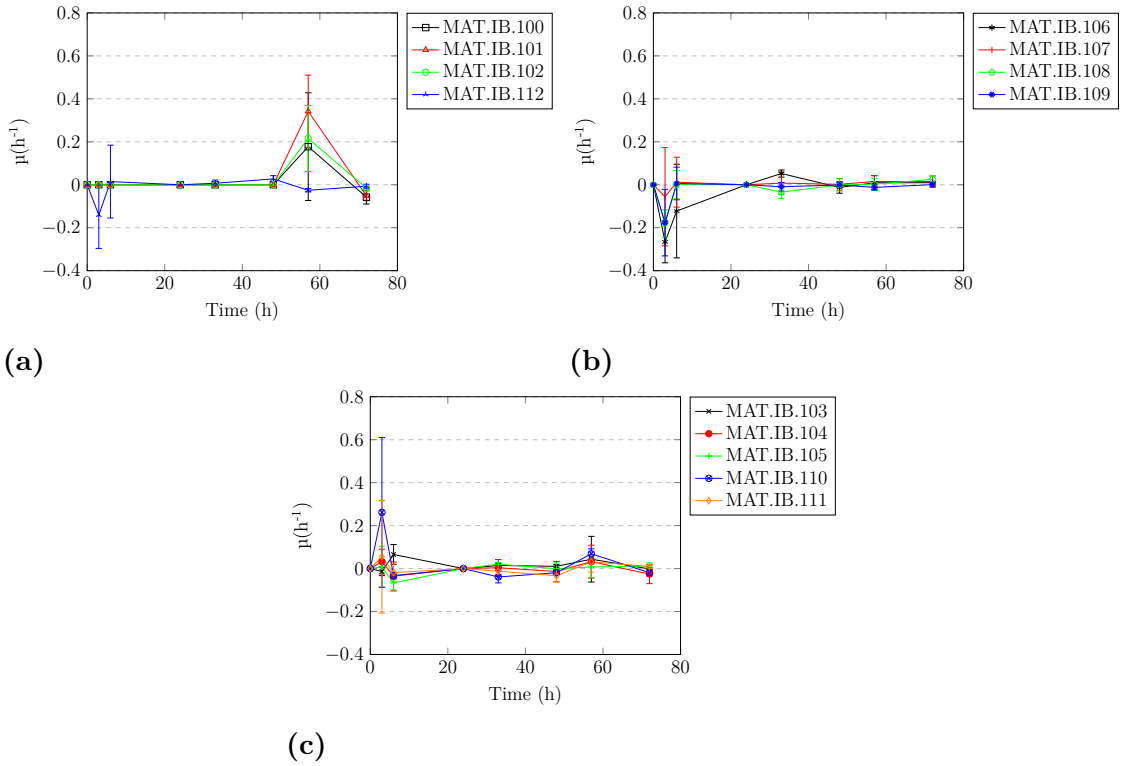


Figure 4.13: Specific growth rate of the yeast with standard deviation during SSCF in all 13 materials. (a) The star points (materials MAT.IB.100-102 and MAT.IB.112). (b) The center points (materials MAT.IB.106-109). (c) The edge points (materials MAT.IB.103-105 and MAT.IB.110-111).

5

Discussion

5.1 Preculture fermentations

The preculture fermentations were mostly similar in terms of offgas CO_2 and DO, with the exception of the first batch. This batch had a delayed and higher first offgas CO_2 peak, indicating that the yeast cells were in the initial sugar consumption phase for longer compared to the subsequent batches (Figure 4.1a). One possible reason for this could be that the preculture fermentation media was wrongly prepared, causing the sugar concentration to be higher in this batch. The ethanol consumption phase was also longer in the first preculture batch. Higher concentrations of sugars could lead to more ethanol produced, assuming the sugar concentrations are not too high, which would cause substrate inhibition.

Similarly to the offgas CO_2, the offgas DO was also slightly delayed in the first preculture batch (Figure 4.1b). As the ethanol consumption phase lasts longer the cells are able to consume oxygen longer.

The harvested cells seemed to be in similar growth phases in all preculture batches thanks to the experimental design. Cells were taken at the same amount of time after the ethanol consumption phase had ended, indicated by the second CO_2 offgas peak.

5.2 Fermentation products and sugars

A maximum concentration of approximately 12.5 g l^{-1} ethanol was produced during SSCF with material MAT.IB.100 after 24 h of fermentation (Figure 4.2a). The ethanol concentration leveled out and even decreased after 24 h of SSCF in all materials with the exception of MAT.IB.102 (Figure 4.2). This is an indication that the ethanol evaporated into the nitrogen gas stream over time. Material MAT.IB.102 showed the highest final titer of approximately 10 g l^{-1} ethanol. It is possible that there were less ethanol evaporation in these shake flasks but it is also possible that

the produced ethanol concentration were high enough to not be as affected by the evaporation. Assuming the evaporation rate is different in each of the shake flask triplicates there would be highly varying results and thus a large standard deviation. The three materials that displayed the highest ethanol titers are materials MAT.IB.100, MAT.IB.102 and material MAT.IB.103. There are quite large standard deviations within the MAT.IB.103 samples. 1 of the 3 shake flasks were run at different times. It could be a possibility that the cells used were in different viability levels since they come from different preculture batches. Material MAT.IB.100 was pretreated with low temperature and long time and materials MAT.IB.102 and MAT.IB.103 were pretreated with high temperature and short time. Materials MAT.IB.100 and MAT.IB.103 have fairly high severity factors (4.36 and 4.20) while MAT.IB.102 has a fairly low severity factor (3.91). This makes it difficult to connect the pretreatment strategy to ethanol titers. The remaining materials display similar trends in ethanol titers where the titer reaches approximately 4 g l^{-1} after 9 h of SSCF after which the ethanol concentration starts to level out and even decrease, possibly due to ethanol evaporation.

The yeast were able to consume both glucose and hemicelluloses to the greatest extent during SSCF with materials MAT.IB.100 and MAT.IB.102 (Figures 4.3a and 4.4a). These materials have some of the lower concentrations of glucose and moderate to high concentrations of xylose (Table A.1). Too high glucose concentrations will inhibit xylose consumption in yeast, this would mean that a lower glucose concentration is favorable for co-fermentation of glucose and xylose which was observed in the SSCF experiments (Figures 4.2a, 4.3a and 4.4a). This could also be observed during SSCF with material MAT.IB.110, which had a fairly high glucose consumption but low hemicellulose consumption (Figure 4.3c and 4.4c). This material has a higher initial glucose concentration compared with materials MAT.IB.100 and MAT.IB.102. Since the hemicellulose consumption in material MAT.IB.110 is lower compared to materials MAT.IB.100 and MAT.IB.102, there could be indications that pretreatment processing methods with moderate temperature and and time could be unfavorable for glucose and xylose co-consumption in SSCF. A way to deal with the issue of too high glucose concentration could be the use of controlled feeding of the substrate by operating in controlled fed-batch mode. This enables high gravity, high WIS substrates to be used during the fermentation. The fact that the hemicellulose was released slower than glucose, and in some cases stayed constant, could be an indication that most of the hemicelluloses are released during pretreatment and not hydrolyzed by the enzyme mixture.

Xylitol is a byproduct of xylose fermentation with yeasts that has the XR/XDH pathway. Xylose is metabolized into xylitol, which is then further utilized in the yeast metabolism via xylulose. Production of xylitol is then a sign of xylose consumption, but with low xylitol conversion. This xylitol excretion is due to an intracellular redox imbalance caused by the different co-enzyme specificities of XR and XDH. Xylitol production could thus be a sign of xylose consumption where the cells are unable to further metabolize xylitol. The xylitol titers are fairly similar in all materials (Figure 4.5). Xylitol starts to be produced at 6 h into the SSCF. It is

at around this time, 6 to 9 h, that the hemicellulose starts to be consumed in the cases where it decreases. This could an indication of xylose consumption. However, all materials show some xylitol production but not all materials show hemicellulose consumption.

Cellobiose is initially released during the first 3 h of SSCF after which it decreases and then released again after 24 h (Figure 4.6). The cellobiose increase after 24 h could be a sign that the β-glucosidase in the enzyme mixture could not cope with the inhibitor levels.

There was a significant unidentified peak observed during HPLC analysis (Figure 4.7). This could to be some kind of fermentation byproduct that was produced. The highest peak areas was observed in materials MAT.IB.106, MAT.IB.107 and MAT.IB.109. These materials also had some of the lower ethanol productions and sugar consumptions. This could be an indication that the unknown compound was some form byproduct that the cells excreted during stress from low sugar environments or environments where the sugars were unavailable to the yeast. The peak areas for Materials MAT.IB.100 and MAT.IB.102 are some of the lower areas. Since these materials have higher ethanol titers, it is also a possibility that the peak could due to a detoxification process.

The highest ethanol yields were observed during SSCF with materials MAT.IB.100 and MAT.IB.102 (Figures 4.8). These materials were pretreated under different conditions. MAT.IB.100 was pretreated under low temerature and long time while MAT.IB.102 was pretreated under high temperature and short time. The severity factor of material MAT.IB.100 (4.36) is also higher than material MAT.IB.102 (3.91). Like in the case of ethanol titer, it is difficult to connect pretreatment strategies to the ethanol yield or rate. The volumetric ethanol production rates does not seem to correlate well with the overall ethanol yields (Figures 4.8 and 4.9). The rates during the first 9 h of the experiments were not noticeably higher in materials MAT.IB.100 and 102, which had the higher yields. This could be due to longer fermentation times. This is also observable in the CFU results, where the calculated CFU started to decrease later in materials MAT.IB.100 and 102 compared to the other materials.

5.3 Water insoluble solids

The WIS of all materials stayed consistently at around 10 % with the exception of MAT.IB.103, which increased by approximately 6.5 % (Figure 4.10). This material has one of the lowest inherent WIS values (Table A.1). The WIS levels of approximately 10 % shows that the media preparation was successful since the aiming point was to have 10 % in all shake flasks. The slight increase in some cases could be due to the consumption of some of the liquid fraction in the media since the solid fraction is unavailable for the cells to ferment. It is possible it could be because of yeast growth or possibly due to uneven sampling. Another reason could be that the

increase in WIS was due to evaporation.

5.4 Cell viability

Cell counting in the Neubauer chamber indicated that the cells were able to, at the very least, survive in the pre-treated spruce slurry (Figure 4.11, Table E.1). However, the CFUs did decrease to low values during the first 24 h (Figure 4.12, Table E.1). This indicates that cells able to survive after 24 h did not spend much, if any, energy on growth. This could be due to the cells losing their ability to grow in fresh YPD media when removed from the spruce slurry. At this point the cells were viable but not culturable. Since the ethanol concentration and sugar consumption rate had started to level out at this point, the cells likely spent most energy on maintenance and survival or they had become inactivated. The materials that do have CFUs greater than zero after 74 h are materials MAT.IB.100-103 and MAT.IB.110. These materials also has some of the higher sugar consumption rates and/or ethanol production rates. Materials MAT.IB.100 and MAT.IB.102 had high CFUs for up to 48 h of SSCF. This is most likely due to the fact that samples from these material were not diluted enough before 48 h and the amount of colonies were then too numerous to count. It seems likely that if samples from these materials would have been diluted more before 48 h of SSCF, the CFU would have followed a similar trend as the other materials. The large standard deviation associated with the cell concentration renders it difficult to observe any distinct trends differentiating the materials. This may be due to the difficulty to accurately count and differentiate cells from lignocellulosic media. The samples could have been diluted further. However, this would cause too few cells to be counted in the chamber. An example of this is samples from materials MAT.IB.100-102 which were diluted more than the other samples from the other materials (Figure 4.11a). The materials that do seem to yield slightly higher cell concentrations, MAT.IB.104-106, have in general higher initial sugar concentrations, such as glucose and xylose (Table A.1). These materials also have fairly high concentrations of most inhibitors found in the media. This could be an indication that the robustness in the yeast cells makes sugar concentration more important than inhibitor concentration in terms of cell viability. Weak acids in lignocellulosic media have previously been shown to reduce the amount of available ATP for biomass formation [19]. This among with other various inhibitors found in the pretreated spruce tips, needles and branches have been shown to inhibit growth overall. This can be observed in the reduced CFUs in all materials.

The growth rates fluctuated between negative and positive values and were in general low (Figure 4.13). This indicates that the yeast had a difficulties to grow in the media. However, the yeast cells harvested from the preculture batches were already in the stationary phase before transfer to the SSCF shake flask system. Since the cells were in stationary phase the growth rate would then be low. The materials that displayed the highest growth rates are materials MAT.IB.100-102. These materials

also enable some of the higher sugar consumptions.

5.5 Pretreatment conditions

Materials MAT.IB.100 and 102 displayed the highest titers, rates and yields (TRY) as well as cell viability. Material MAT.IB.100 were pretreated under low temperature and long time while material MAT.IB.102 were pretreated under high temperature and short time. The severity factor also differed between the two materials. Material MAT.IB.100 had a higher severity factor (4.36) while material MAT.IB.102 had a lower severity factor (3.91). This makes it difficult to make definitive statements about preferable pretreamtment conditions. There are, however, indications that if the pretreatment temperature is high, the process time needs to be low or vice versa to reach higher TRY and cell viability. One reason for the higher TRY in materials MAT.IB.100 and 102 could be due to lower inhibitory effects. This could possibly be observed in the volumetric production rates and CFU counts as it is a possibility that the fermentation times were longer in these materials (Figures 4.9 and 4.12). Another possibility could be the higher release of available sugars in these materials that the yeast were able to ferment. This is as these materials displayed some of the more favorable sugar consumption trends (Figures 4.3 and 4.4).

6

Conclusion

A reliable preculture propagation method was developed that generated enough cell mass which was in a similar physiological state each batch, prior to cell harvest and transfer to a SSCF shake flask system.

Acid-catalyzed steam pretreated spruce tips, needles and branches could be fermented during SSCF in small lab scale experiments. The highest TRY were observed in materials MAT.IB.100 and MAT.IB.102. The yeast were also able to consume the most amount of sugars in these materials. These materials were, however, pretreated under different process conditions. Material MAT.IB.100 were pretreated under low temperature and long time while material MAT.IB.102 were pretreated under high temperature and short time. Furthermore, the severity factor also differs between the two materials. Material MAT.IB.100 has a higher severity factor (4.36) while material MAT.IB.102 has a lower severity factor (3.91). Thsi may be an indication thet thae severity factor puts too much weight in wither time or temperature. This renders it difficult to draw a conclusion connecting pretreatment with fermentability. Further statistical testing is required to make more certain conclusions and recommendations. Some results also display high standard deviation within replicates. This will also increase uncertainty of the results.

The ethanol yield is an important factor in determining the process economy of bioethanol production using SSCF processes [35]. Based on the trends observed, one indication seems to be that either high temperature and short process time or low temperature and long process time could be the most beneficial pretreatment process conditions in terms of final TRY using spruce tips, needles and branches.

One option to further evaluate the fermentability of the pretreated spruce tips, needles and branches could be to perform the SSCF in larger lab scales operating in fed-batch mode. By controlling the feed of substrate, enzymes and cells it is possible to increase the gravity of the media without causing substrate inhibition in the yeast. It is also possible to adapt the yeast cells to the fermentation media in preculture propagations. Another option could be to perform further more rigorous statistical testing that, unfortunately, was omitted due to time constraints in the project.

7

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A

Appendix 1

A.1 Raw material

The raw material was provided and pretreated by the SP Biorefinery Demo Plant (Örnsköldsvik, Sweden) (Table A.1). The 13 different combinations are divided into 3 groups. The star points (materials MAT.IB.100-102 and MAT.IB.112), the center points (MAT.IB.106-109) and the edge points (MAT.IB.103-105 and MAT.IB.110-111).

Table A.1: Composition of steam-preatreated raw materials [53].

Material name	MAT.IB.100*	MAT.IB.101*	MAT.IB.102*	MAT.IB.103***	MAT.IB.104***	MAT.IB.105***	MAT.IB.106**	MAT.IB.107**	MAT.IB.108**	MAT.IB.109**	MAT.IB.110***	MAT.IB.111***	MAT.IB.112*
Pre-treatment variables													
Temperature (°C)	204	214	211	214	204	204	209	209	209	209	200	206	213
Time (min)	20.0	20.0	4.40	7.00	7.00	7.00	13.5	13.5	13.5	13.5	13.5	13.5	22.6
Severity factor	4.36	4.66	3.91	4.20	3.91	3.91	4.34	4.34	4.34	4.34	4.07	4.25	4.68
Total solids (%)	19.8	18.1	21.4	18.7	24.6	23.9	20.0	20.0	22.0	22.3	26.0	24.3	17.9
Water insoluble solids (%)	14.5 ± 1.03	13.9 ± 0.19	15.1 ± 0.35	13.1 ± 0.30	17.2 ± 0.39	16.9 ± 0.30	14.2 ± 0.61	13.8 ± 0.40	15.7 ± 0.80	15.9 ± 0.29	18.7 ± 0.38	17.6 ± 0.37	12.5±0.71
Soluble solids (%)	5.34	4.18	6.34	5.55	7.39	6.96	5.81	6.20	6.26	6.42	7.27	6.74	5.43
Compositional analysis													
Ash content (%)	4.88 ± 0.28	4.89 ± 0.17	4.72 ± 0.35	4.50 ± 0.13	4.87 ± 0.05	4.87 ± 0.20	4.67 ± 0.22	4.51 ± 0.16	5.05 ± 0.08	4.62 ± 0.05	5.01 ± 0.04	$5.03 \pm n.a.$	4.91 ± 0.28
Adjusted pH	5.12	5.11	5.09	5.07	5.08	5.11	5.05	5.02	5.02	5.06	5.04	5.04	5.14
Solid phase													
Cellulose (g (g WIS)$^{-1}$)	27.6 ± 0.66	30.3 ± 0.67	30.0 ± 0.72	32.2 ± 4.07	31.0 ± 0.83	28.0 ± 0.49	25.5 ± 3.58	29.0 ± 1.57	23.0 ± 2.56	23.9 ± 2.98	29.9 ± 0.77	22.9 ± 2.89	25.0 ± 0.83
Hemicelluloses													
Mannan (g (g WIS)$^{-1}$)	1.85 ± 0.04	2.37 ± 0.21	1.94 ± 0.02	1.88 ± 0.05	$n.a. \pm n.a.$	$n.a. \pm n.a.$	3.70 ± 0.01	3.74 ± 0.00	3.68 ± 0.02	3.67 ± 0.00	3.28 ± 0.04	$3.23 \pm n.a.$	$n.a. \pm n.a.$
Galactan (g (g WIS)$^{-1}$)	1.28 ± 0.03	1.47 ± 0.09	1.30 ± 0.00	1.28 ± 0.05	$n.a. \pm n.a.$	$n.a. \pm n.a.$	0.68 ± 0.01	0.68 ± 0.02	0.63 ± 0.01	0.64 ± 0.00	0.64 ± 0.02	$n.a. \pm n.a.$	$n.a. \pm n.a.$
Arabinan (g (g WIS)$^{-1}$)	1.40 ± 0.02	1.42 ± 0.02	1.44 ± 0.01	1.34 ± 0.01	$n.a. \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$	2.81 ± 0.01	2.83 ± 0.03	1.04 ± 0.02	$n.a. \pm n.a.$	$n.a. \pm n.a.$
Xylan (g (g WIS)$^{-1}$)	2.96 ± 0.01	3.36 ± 0.16	3.00 ± 0.02	3.09 ± 0.06	$n.a. \pm n.a.$	$n.a. \pm n.a.$	2.85 ± 0.06	2.90 ± 0.04	2.80 ± 0.03	2.77 ± 0.01	2.84 ± 0.06	$2.77 \pm n.a.$	$n.a. \pm n.a.$
Liquid phase													
Hemicelluloses													
Rhamnose (g l^{-1})	0.58 ± 0.06	0.44 ± 0.05	0.75 ± 0.03	0.77 ± 0.03	0.70 ± 0.05	0.68 ± 0.02	0.59 ± 0.04	0.48 ± 0.02	$0.41 \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$
Arabinose (g l^{-1})	4.67 ± 0.13	2.98 ± 0.07	6.19 ± 0.07	7.48 ± 0.11	5.88 ± 0.50	5.76 ± 0.12	5.17 ± 0.54	4.27 ± 0.07	2.88 ± 0.83	2.30 ± 0.63	6.68 ± 1.89	2.51 ± 0.58	$2.60 \pm n.a.$
Galactose (g l^{-1})	5.61 ± 0.11	3.53 ± 0.10	6.45 ± 0.08	7.03 ± 0.10	7.53 ± 0.70	7.06 ± 0.13	6.58 ± 0.66	5.64 ± 0.41	4.89 ± 1.09	4.61 ± 1.00	7.84 ± 2.02	4.47 ± 0.87	$3.92 \pm n.a.$
Xylose (g l^{-1})	3.20 ± 0.09	1.72 ± 0.06	4.30 ± 0.09	6.11 ± 0.14	4.23 ± 0.35	3.89 ± 0.07	4.16 ± 0.45	3.57 ± 0.22	2.05 ± 0.43	1.92 ± 0.37	4.78 ± 1.18	2.17 ± 0.36	$2.21 \pm n.a.$
Mannose (g l^{-1})	5.65 ± 0.09	3.52 ± 0.13	7.09 ± 0.12	8.65 ± 0.18	7.75 ± 0.72	7.58 ± 0.14	7.00 ± 0.72	5.72 ± 0.42	4.54 ± 0.99	4.08 ± 0.89	8.66 ± 2.18	4.12 ± 0.74	$3.78 \pm n.a.$
Glucose													
Glucose (g l^{-1}) (IC)	13.5 ± 0.24	10.5 ± 0.35	16.8 ± 0.01	12.0 ± 0.15	20.6 ± 1.94	19.8 ± 0.36	14.7 ± 1.49	14.2 ± 0.97	17.9 ± 0.10	17.3 ± 3.86	18.6 ± 0.25	12.1 ± 0.38	$10.9 \pm n.a.$
Glucose (g l^{-1}) (HPLC)	13.0 ± 1.11	12.7 ± 0.13	20.4 ± 1.25	12.1 ± 0.79	22.6 ± 1.33	21.9 ± 0.36	16.4 ± 0.16	15.2 ± 0.14	19.0 ± 0.35	22.7 ± 0.52	20.2 ± 0.90	20.0 ± 0.99	11.7 ± 0.30
Potential inhibitors													
Acetic acid (g l^{-1}) (HPLC)	3.35 ± 0.28	3.70 ± 0.08	4.02 ± 0.17	2.93 ± 0.24	4.83 ± 0.29	4.76 ± 0.02	3.99 ± 0.04	3.78 ± 0.08	4.52 ± 0.07	4.92 ± 0.12	5.00 ± 0.14	5.23 ± 0.25	3.65 ± 0.09
Formic acid (g l^{-1}) (HPLC)	1.19 ± 0.06	0.98 ± 0.01	0.97 ± 0.10	0.50 ± 0.05	1.34 ± 0.07	1.19 ± 0.07	0.97 ± 0.06	1.48 ± 0.08	1.60 ± 0.06	2.38 ± 0.03	1.02 ± 0.02	2.08 ± 0.09	1.10 ± 0.05
Levulinic acid (g l^{-1}) (HPLC)	1.20 ± 0.08	1.50 ± 0.03	1.08 ± 0.04	0.37 ± 0.03	1.42 ± 0.11	1.51 ± 0.01	1.09 ± 0.02	1.66 ± 0.02	2.11 ± 0.05	3.45 ± 0.07	1.15 ± 0.05	2.9 ± 0.16	1.59 ± 0.00
Glycerol (g l^{-1}) (HPLC)	0.30 ± 0.01	0.43 ± 0.01	0.41 ± 0.02	0.41 ± 0.02	0.45 ± 0.04	0.44 ± 0.01	0.36 ± 0.00	0.18 ± 0.00	0.41 ± 0.01	0.32 ± 0.01	0.60 ± 0.03	0.45 ± 0.02	0.39 ± 0.01
HMF (g l^{-1}) (HPLC)	3.13 ± 0.27	4.32 ± 0.07	3.42 ± 0.20	1.45 ± 0.08	4.33 ± 0.21	4.35 ± 0.08	3.66 ± 0.02	3.52 ± 0.06	5.11 ± 0.08	5.05 ± 0.13	3.56 ± 0.15	5.33 ± 0.25	4.25 ± 0.10
Furfural (g l^{-1}) (HPLC)	1.74 ± 0.15	1.82 ± 0.05	1.96 ± 0.14	0.93 ± 0.04	2.43 ± 0.15	2.42 ± 0.11	1.86 ± 0.03	1.76 ± 0.10	2.42 ± 0.03	2.52 ± 0.04	2.17 ± 0.08	2.31 ± 0.17	1.61 ± 0.01
Vanillin (g l^{-1}) (HPLC)	$n.a. \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$	0.04 ± 0.04	$n.a. \pm n.a.$	$0.21 \pm n.a.$	$n.a. \pm n.a.$	$0.34 \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$	$n.a. \pm n.a.$

* Star points of the design of experiments
** Center points of the design of experiments
*** Edge points of the design of experiments

B

Appendix 2

B.1 High-performance liquid chromatography standards

The single standards and standard mixtures were serially diluted into 1:1, 1:2, 1:4, 1:8, 1:32 and 1:64 dilutions. (Table B.1)

Table B.1: Single standards and standard mixtures used to identify compounds in the HPLC analysis.

HPLC standards	
Substance name	Concentration (g l^{-1})
Single standards	
Glucose	25
Galactose	25
Mannose	25
Xylose	25
Glycerol	5
HMF	10
Furfural	10
Dihydrated sodium citrate	15
Xylitol	10
Arabinose	25
Rhamnose	25
Fructose	25
Cellobiose	25
Ethanol	25
Levulinic acid	2.5
Sodium acetate	12.5
Formic acid	5
Ferulic acid	0.5
Sugar mix	
Glucose	25
Galactose	25
Mannose	25
Xylose	25
Ethanol	25
Arabinose	25
Rhamnose	25
Xylitol	10
Cellobiose	25
Glycerol	5
Acid mix	
Levulinic acid	2.5
Sodium acetate	12.5
Dihydrated sodium citrate	15
Formic acid	5
Lactic acid	5
Phenolics mix	
HMF	10
Furfural	10

C

Appendix 3

C.1 Fermentation products and sugars

The concentrations of the measured fermentation products and inhibitors was obtained via HPLC measurements (C.1). Values below the limit of detection are listed as 0. These concentrations correspond to a peak area lower than a 1:64 dilution of the standard used to identify the peak.

The ethanol yields and rates were calculated using equations 3.8-3.10 (Table C.2).

Table C.1: Concentrations of the measured fermentation products and sugars with standard deviation.

Fermentation products and sugars													
Time (h)	MAT.IB.100	MAT.IB.101	MAT.IB.102	MAT.IB.103	MAT.IB.104	MAT.IB.105	MAT.IB.106	MAT.IB.107	MAT.IB.108	MAT.IB.109	MAT.IB.110	MAT.IB.111	MAT.IB.112
Ethanol (g l^{-1})													
0.00	1.18 ± 0.03	1.20 ± 0.03	1.17 ± 0.01	1.20 ± 0.02	1.15 ± 0.02	1.13 ± 0.01	0.00 ± 1.47	0.00 ± 0.02	0.00 ± 0.05	0.00 ± 0.05	0.00 ± 0.04	0.00 ± 0.01	0.00 ± 0.01
3.00	0.43 ± 0.14	0.13 ± 0.02	0.57 ± 0.06	0.15 ± 0.32	0.12 ± 0.15	0.03 ± 0.09	0.00 ± 0.21	0.00 ± 0.09	0.00 ± 0.23	0.00 ± 0.15	0.19 ± 0.10	0.00 ± 0.05	0.00 ± 0.20
6.00	3.42 ± 0.25	1.71 ± 0.36	2.39 ± 0.37	1.11 ± 1.04	1.27 ± 0.29	1.08 ± 0.30	0.56 ± 0.23	1.07 ± 0.40	0.85 ± 0.31	0.73 ± 0.37	1.66 ± 0.33	0.86 ± 0.19	0.47 ± 0.30
9.00	4.93 ± 0.26	3.52 ± 0.24	4.39 ± 0.36	3.70 ± 0.63	3.80 ± 0.18	3.27 ± 0.40	3.06 ± 0.16	3.32 ± 0.18	3.41 ± 0.13	3.24 ± 0.17	3.77 ± 0.63	3.47 ± 0.06	3.15 ± 0.14
24.00	12.4 ± 5.26	1.26 ± 0.53	5.90 ± 1.62	4.53 ± 4.44	3.42 ± 0.45	3.33 ± 0.75	2.62 ± 0.48	3.02 ± 0.41	3.41 ± 0.23	2.91 ± 0.61	4.20 ± 1.38	3.75 ± 0.03	2.97 ± 0.43
48.00	7.58 ± 3.74	2.85 ± 0.58	7.19 ± 2.68	6.04 ± 2.91	3.15 ± 0.50	3.04 ± 0.99	3.01 ± 0.73	2.43 ± 0.39	2.37 ± 0.17	2.19 ± 0.29	3.92 ± 1.66	3.57 ± 0.48	2.77 ± 0.57
72.00	5.62 ± 2.57	2.08 ± 0.64	10.1 ± 1.62	2.33 ± 0.65	1.78 ± 0.51	1.97 ± 0.92	1.55 ± 0.63	1.20 ± 0.07	1.33 ± 0.03	1.85 ± 0.79	2.88 ± 0.94	2.22 ± 0.48	1.80 ± 0.60
Glucose (g l^{-1})													
0.00	10.6 ± 0.59	10.6 ± 0.17	13.5 ± 0.50	11.6 ± 0.03	15.3 ± 0.26	13.7 ± 0.09	16.7 ± 4.69	21.1 ± 0.30	22.7 ± 1.40	25.7 ± 3.80	20.1 ± 0.12	20.4 ± 0.13	17.0 ± 0.30
3.00	16.7 ± 0.60	17.7 ± 0.65	22.6 ± 1.63	20.1 ± 1.49	26.5 ± 0.27	25.2 ± 1.54	22.8 ± 0.65	20.9 ± 1.71	18.1 ± 0.10	24.3 ± 5.22	21.8 ± 0.50	23.3 ± 2.78	19.4 ± 0.50
6.00	15.1 ± 0.98	20.1 ± 1.13	21.5 ± 1.73	22.1 ± 0.75	24.7 ± 0.35	24.8 ± 0.84	22.7 ± 0.63	21.1 ± 0.93	19.8 ± 2.46	23.8 ± 6.31	20.7 ± 0.37	20.7 ± 0.46	20.2 ± 0.63
9.00	7.11 ± 0.73	9.95 ± 0.23	11.8 ± 0.78	12.3 ± 1.03	14.4 ± 0.42	14.2 ± 0.53	12.8 ± 0.28	11.4 ± 0.31	11.1 ± 1.04	13.1 ± 3.11	11.9 ± 0.20	12.3 ± 0.06	11.9 ± 0.41
24.00	0.29 ± 1.04	21.6 ± 2.66	18.6 ± 3.32	19.3 ± 7.24	16.6 ± 0.77	17.0 ± 1.29	16.8 ± 0.33	13.3 ± 0.31	12.5 ± 1.37	15.6 ± 2.92	12.2 ± 0.87	15.4 ± 0.21	14.3 ± 0.46
48.00	1.70 ± 0.02	15.6 ± 1.06	5.97 ± 3.45	7.48 ± 4.23	19.4 ± 0.55	20.8 ± 0.98	18.9 ± 1.76	17.6 ± 3.74	15.9 ± 1.24	17.7 ± 1.52	11.8 ± 3.03	17.9 ± 0.67	17.0 ± 0.57
72.00	1.54 ± 0.09	16.5 ± 1.46	1.84 ± 0.03	3.39 ± 1.61	22.5 ± 0.60	23.5 ± 1.74	23.6 ± 1.24	19.5 ± 1.11	17.4 ± 2.09	22.0 ± 3.41	8.24 ± 5.29	18.9 ± 0.86	18.8 ± 0.70
Hemicellulose (g l^{-1})													
0.00	3.68 ± 0.17	3.03 ± 0.06	4.70 ± 0.04	6.08 ± 0.10	4.79 ± 0.08	4.27 ± 0.02	4.74 ± 0.47	5.02 ± 0.05	3.21 ± 0.12	2.68 ± 0.46	5.10 ± 0.04	2.26 ± 0.01	2.04 ± 0.06
3.00	3.59 ± 0.11	2.10 ± 0.03	4.76 ± 0.10	8.00 ± 0.88	5.43 ± 0.03	4.20 ± 0.29	6.07 ± 0.22	4.90 ± 0.41	1.81 ± 0.27	2.15 ± 0.50	5.86 ± 0.13	2.96 ± 0.54	2.56 ± 0.05
6.00	3.00 ± 0.12	2.61 ± 0.15	4.58 ± 0.14	9.14 ± 0.38	4.28 ± 0.06	4.16 ± 0.40	4.77 ± 0.14	4.88 ± 0.05	2.56 ± 0.16	1.78 ± 0.22	5.33 ± 0.03	2.03 ± 0.37	2.38 ± 0.06
9.00	3.06 ± 0.10	2.94 ± 0.06	4.36 ± 0.11	6.13 ± 0.07	4.50 ± 0.05	4.23 ± 0.06	4.19 ± 0.07	4.09 ± 0.02	3.19 ± 0.04	2.99 ± 0.26	4.60 ± 0.10	3.09 ± 0.13	3.16 ± 0.04
24.00	0.00 ± 0.89	1.90 ± 0.16	4.78 ± 0.25	7.13 ± 1.03	4.53 ± 0.11	4.27 ± 0.14	4.77 ± 0.14	4.09 ± 0.04	3.15 ± 0.08	3.04 ± 0.04	4.48 ± 0.18	3.11 ± 0.04	3.15 ± 0.06
48.00	1.33 ± 0.01	3.00 ± 0.15	3.16 ± 1.04	4.32 ± 1.56	4.52 ± 0.11	4.44 ± 0.42	4.20 ± 0.87	3.18 ± 0.19	3.59 ± 0.51	4.51 ± 0.46	4.31 ± 0.45	3.12 ± 0.04	3.27 ± 0.13
72.00	1.06 ± 0.01	2.76 ± 0.22	1.46 ± 0.18	3.16 ± 1.04	4.44 ± 0.21	4.37 ± 0.64	5.05 ± 0.56	4.03 ± 0.35	3.12 ± 0.11	3.25 ± 0.46	3.22 ± 1.28	2.90 ± 0.08	3.09 ± 0.20
Xylitol (g l^{-1})													
0.00	0.22 ± 0.00	0.24 ± 0.01	0.31 ± 0.00	0.21 ± 0.01	0.32 ± 0.01	0.28 ± 0.01	0.00 ± 1.26	0.00 ± 0.02	0.00 ± 0.04	0.00 ± 0.05	0.00 ± 0.04	0.00 ± 0.04	0.00 ± 0.02
3.00	0.00 ± 0.00	0.00 ± 0.02	0.00 ± 0.05	0.00 ± 0.04	0.00 ± 0.05	0.00 ± 0.05	0.00 ± 0.02	0.00 ± 0.01	0.00 ± 0.03	0.00 ± 0.06	0.00 ± 0.03	0.00 ± 0.12	0.00 ± 0.09
6.00	0.00 ± 0.03	0.00 ± 0.02	0.00 ± 0.02	0.00 ± 0.00	0.00 ± 0.03	0.00 ± 0.04	0.00 ± 0.02	0.00 ± 0.03	0.00 ± 0.09	0.00 ± 0.06	0.00 ± 0.01	0.00 ± 0.04	0.00 ± 0.04
9.00	0.33 ± 0.00	0.34 ± 0.01	0.37 ± 0.01	0.32 ± 0.00	0.46 ± 0.01	0.44 ± 0.00	0.38 ± 0.01	0.34 ± 0.01	0.43 ± 0.01	0.47 ± 0.00	0.33 ± 0.01	0.43 ± 0.01	0.36 ± 0.01
24.00	0.00 ± 0.00	0.00 ± 0.04	0.00 ± 0.06	0.00 ± 0.01	0.50 ± 0.01	0.45 ± 0.02	0.37 ± 0.00	0.35 ± 0.01	0.46 ± 0.01	0.49 ± 0.01	0.36 ± 0.01	0.46 ± 0.01	0.39 ± 0.01
48.00	0.00 ± 0.00	0.36 ± 0.01	0.37 ± 0.03	0.32 ± 0.00	0.44 ± 0.01	0.41 ± 0.01	0.39 ± 0.06	0.48 ± 0.03	0.41 ± 0.05	0.35 ± 0.01	0.35 ± 0.00	0.44 ± 0.01	0.36 ± 0.01
72.00	0.00 ± 0.00	0.21 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.28 ± 0.01	0.28 ± 0.01	0.22 ± 0.01	0.21 ± 0.00	0.33 ± 0.01	0.36 ± 0.05	0.20 ± 0.00	0.28 ± 0.01	0.22 ± 0.01
Cellobiose (g l^{-1})													
0.00	0.00 ± 0.00	0.00 ± 0.00	1.79 ± 0.05	0.00 ± 0.00	1.90 ± 0.02	1.78 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.95 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3.00	0.00 ± 0.00	0.00 ± 0.00	2.98 ± 0.33	2.94 ± 0.40	3.61 ± 0.06	3.20 ± 0.26	3.30 ± 0.18	3.18 ± 0.16	0.00 ± 0.00	4.85 ± 0.00	3.43 ± 0.04	3.81 ± 0.00	2.63 ± 0.38
6.00	2.41 ± 0.12	2.67 ± 0.27	3.16 ± 0.31	4.05 ± 0.28	3.63 ± 0.06	3.67 ± 0.12	3.40 ± 0.56	3.90 ± 0.25	2.72 ± 0.37	3.72 ± 1.68	3.73 ± 0.04	2.67 ± 0.01	3.46 ± 0.85
9.00	0.00 ± 0.00	1.91 ± 0.03	2.11 ± 0.03	2.24 ± 0.08	2.26 ± 0.01	2.24 ± 0.03	2.16 ± 0.12	2.24 ± 0.10	2.05 ± 0.04	2.13 ± 0.33	2.24 ± 0.03	2.10 ± 0.06	2.19 ± 0.22
24.00	1.59 ± 0.10	3.29 ± 0.80	3.53 ± 1.02	4.40 ± 0.57	0.13 ± 0.02	0.25 ± 0.18	0.26 ± 0.29	0.23 ± 0.23	0.02 ± 0.12	0.29 ± 0.25	0.05 ± 0.05	0.39 ± 0.06	0.03 ± 0.28
48.00	1.79 ± 0.04	2.32 ± 0.13	2.01 ± 0.29	2.18 ± 0.30	2.57 ± 0.07	2.75 ± 0.05	2.56 ± 0.13	2.66 ± 0.15	2.42 ± 0.10	3.02 ± 0.38	2.42 ± 0.10	2.73 ± 0.06	2.41 ± 0.15
72.00	1.56 ± 0.05	2.08 ± 0.04	1.70 ± 0.22	1.83 ± 0.25	2.50 ± 0.09	2.66 ± 0.10	2.76 ± 0.36	2.75 ± 0.54	2.39 ± 0.01	2.65 ± 0.21	2.09 ± 0.25	2.45 ± 0.13	2.29 ± 0.11
Unknown (HPLC peak area)													
0.00	16870 ± 713	13776 ± 0	12854 ± 1621	64978 ± 25130	83908 ± 48695	92437 ± 41428	83463 ± 17937	31730 ± 2682	21137 ± 6591	143965 ± 2086	104677 ± 0	25935 ± 0	0 ± 0
3.00	207850 ± 23387	413982 ± 61706	262078 ± 23934	595127 ± 161760	267702 ± 21851	374818 ± 93008	596740 ± 102002	414180 ± 8651	295662 ± 47317	374445 ± 42052	422244 ± 67901	232450 ± 23162	368563 ± 45083
6.00	666224 ± 18627	1051131 ± 33332	664776 ± 28683	1310828 ± 132338	728229 ± 10612	858900 ± 10402	1221388 ± 186433	1068255 ± 80055	737228 ± 89393	792069 ± 72822	983908 ± 19037	568922 ± 17316	854337 ± 123069
9.00	945832 ± 23310	1253243 ± 33508	945607 ± 28437	1534271 ± 220570	1062420 ± 24887	1078366 ± 43595	1649899 ± 449142	1689421 ± 320928	1212024 ± 67973	1314351 ± 64591	1431514 ± 25340	805424 ± 8605	1034027 ± 124529
24.00	1670512 ± 54396	1921038 ± 37459	1553893 ± 11131	2163895 ± 179998	1830290 ± 8471	1878436 ± 183481	2644040 ± 1014915	3129806 ± 908239	1997273 ± 376333	2219122 ± 63453	2090091 ± 48171	1063459 ± 102587	1364152 ± 322629
48.00	1634890 ± 89461	1952327 ± 34843	1687151 ± 69448	2298127 ± 223013	1959359 ± 54403	2042602 ± 350667	1972915 ± 127286	2368107 ± 589655	2709662 ± 161263	4134036 ± 943473	2356115 ± 148904	1048531 ± 50295	1488880 ± 478728
72.00	1662928 ± 183013	1818028 ± 78935	1725208 ± 109869	2547404 ± 349040	1975893 ± 70242	2098877 ± 439668	3207155 ± 1497922	3787427 ± 1002443	2317921 ± 583179	2766972 ± 688884	2447735 ± 190883	1010382 ± 52339	1434077 ± 432212

Table C.2: Calculated ethanol yields and volumetric ethanol production rates with standard deviation

Ethanol yields and rates													
Time (h)	MAT.IB.100	MAT.IB.101	MAT.IB.102	MAT.IB.103	MAT.IB.104	MAT.IB.105	MAT.IB.106	MAT.IB.107	MAT.IB.108	MAT.IB.109	MAT.IB.110	MAT.IB.111	MAT.IB.112
Ethanol yield on glucose (g ethanol (g glucose)$^{-1}$)													
0	0.023 ± 0.001	0.024 ± 0.001	0.022 ± 0.000	0.023 ± 0.001	0.021 ± 0.001	0.021 ± 0.000	-0.004 ± 0.036	-0.030 ± 0.000	-0.029 ± 0.001	-0.027 ± 0.003	-0.030 ± 0.001	-0.031 ± 0.000	-0.032 ± 0.000
3	0.009 ± 0.003	0.003 ± 0.000	0.011 ± 0.001	0.003 ± 0.008	0.002 ± 0.003	0.001 ± 0.002	-0.004 ± 0.006	-0.001 ± 0.002	-0.006 ± 0.004	-0.001 ± 0.003	0.003 ± 0.002	-0.002 ± 0.001	-0.006 ± 0.004
6	0.068 ± 0.007	0.034 ± 0.009	0.045 ± 0.009	0.022 ± 0.025	0.023 ± 0.007	0.020 ± 0.007	0.008 ± 0.006	0.018 ± 0.008	0.014 ± 0.006	0.011 ± 0.007	0.028 ± 0.007	0.014 ± 0.004	0.008 ± 0.006
9	0.098 ± 0.007	0.070 ± 0.006	0.082 ± 0.009	0.072 ± 0.015	0.069 ± 0.004	0.061 ± 0.009	0.054 ± 0.011	0.054 ± 0.003	0.054 ± 0.001	0.049 ± 0.006	0.063 ± 0.013	0.058 ± 0.001	0.055 ± 0.003
24	0.245 ± 0.127	0.025 ± 0.013	0.111 ± 0.038	0.088 ± 0.106	0.062 ± 0.010	0.062 ± 0.017	0.051 ± 0.018	0.049 ± 0.008	0.054 ± 0.006	0.045 ± 0.013	0.070 ± 0.028	0.062 ± 0.001	0.052 ± 0.009
48	0.150 ± 0.090	0.056 ± 0.014	0.135 ± 0.063	0.117 ± 0.069	0.057 ± 0.012	0.057 ± 0.023	0.062 ± 0.001	0.040 ± 0.008	0.038 ± 0.003	0.033 ± 0.003	0.065 ± 0.034	0.059 ± 0.010	0.049 ± 0.012
72	0.111 ± 0.062	0.041 ± 0.016	0.189 ± 0.039	0.045 ± 0.016	0.032 ± 0.012	0.037 ± 0.021	0.020 ± 0.003	0.020 ± 0.001	0.021 ± 0.001	0.028 ± 0.016	0.048 ± 0.019	0.037 ± 0.010	0.032 ± 0.013
Ethanol yield on glucose and hemicellulose (g ethanol(g glucose and hemicellulose)$^{-1}$)													
0	0.020 ± 0.001	0.021 ± 0.001	0.019 ± 0.000	0.019 ± 0.000	0.018 ± 0.001	0.018 ± 0.000	-0.003 ± 0.031	-0.026 ± 0.000	-0.026 ± 0.001	-0.025 ± 0.002	-0.026 ± 0.001	-0.028 ± 0.000	-0.029 ± 0.000
3	0.007 ± 0.003	0.002 ± 0.000	0.009 ± 0.001	0.002 ± 0.006	0.002 ± 0.003	0.000 ± 0.002	-0.004 ± 0.005	-0.001 ± 0.002	-0.005 ± 0.004	-0.001 ± 0.003	0.003 ± 0.002	-0.001 ± 0.001	-0.005 ± 0.004
6	0.059 ± 0.006	0.030 ± 0.008	0.038 ± 0.008	0.018 ± 0.021	0.020 ± 0.006	0.017 ± 0.006	0.007 ± 0.005	0.015 ± 0.007	0.012 ± 0.005	0.010 ± 0.007	0.024 ± 0.006	0.013 ± 0.003	0.007 ± 0.006
9	0.085 ± 0.006	0.061 ± 0.005	0.071 ± 0.008	0.060 ± 0.012	0.059 ± 0.004	0.053 ± 0.008	0.047 ± 0.009	0.047 ± 0.003	0.049 ± 0.001	0.045 ± 0.005	0.054 ± 0.011	0.052 ± 0.001	0.050 ± 0.003
24	0.212 ± 0.110	0.022 ± 0.011	0.095 ± 0.033	0.073 ± 0.088	0.053 ± 0.009	0.054 ± 0.015	0.044 ± 0.015	0.043 ± 0.007	0.049 ± 0.005	0.040 ± 0.012	0.061 ± 0.025	0.056 ± 0.001	0.047 ± 0.008
48	0.129 ± 0.078	0.049 ± 0.012	0.116 ± 0.054	0.098 ± 0.057	0.049 ± 0.010	0.049 ± 0.019	0.054 ± 0.000	0.034 ± 0.007	0.034 ± 0.003	0.030 ± 0.003	0.057 ± 0.030	0.053 ± 0.009	0.044 ± 0.011
72	0.096 ± 0.054	0.036 ± 0.014	0.162 ± 0.033	0.038 ± 0.013	0.028 ± 0.010	0.032 ± 0.018	0.017 ± 0.003	0.017 ± 0.001	0.019 ± 0.001	0.026 ± 0.015	0.042 ± 0.017	0.033 ± 0.009	0.028 ± 0.012
Volumetric ethanol production rate (g l^{-1}h^{-1})													
3	0.374 ± 0.054	0.085 ± 0.074	0.202 ± 0.077	-0.015 ± 0.208	0.020 ± 0.055	-0.008 ± 0.061	0.130 ± 0.301	0.480 ± 0.085	0.440 ± 0.057	0.418 ± 0.069	0.574 ± 0.074	0.454 ± 0.035	0.383 ± 0.059
6	0.751 ± 0.032	0.565 ± 0.047	0.637 ± 0.062	0.591 ± 0.101	0.612 ± 0.010	0.541 ± 0.086	0.545 ± 0.011	0.563 ± 0.020	0.628 ± 0.055	0.549 ± 0.006	0.597 ± 0.118	0.595 ± 0.015	0.579 ± 0.012
9	0.503 ± 0.117	0.478 ± 0.057	0.573 ± 0.023	0.728 ± 0.089	0.699 ± 0.035	0.609 ± 0.130	0.689 ± 0.049	0.623 ± 0.092	0.712 ± 0.088	0.692 ± 0.073	0.591 ± 0.119	0.729 ± 0.049	0.741 ± 0.052
24	0.229 ± 0.231	-0.067 ± 0.022	0.083 ± 0.085	0.058 ± 0.160	-0.020 ± 0.015	-0.002 ± 0.022	-0.012 ± 0.024	-0.022 ± 0.017	-0.017 ± 0.011	-0.025 ± 0.011	0.013 ± 0.047	0.008 ± 0.007	-0.011 ± 0.023
48	-0.141 ± 0.073	0.017 ± 0.028	0.088 ± 0.013	-0.046 ± 0.127	-0.034 ± 0.005	-0.028 ± 0.011	-0.022 ± 0.025	-0.038 ± 0.011	-0.043 ± 0.007	-0.022 ± 0.005	-0.028 ± 0.036	-0.032 ± 0.012	-0.024 ± 0.010

D

Appendix 4

D.1 Water insoluble solids

WIS was calculated as total percent of solids using Equation 3.11 (Table D.1).

Table D.1: WIS contents at start and end of fermentation expressed as percent of total solids for all 13 materials with standard deviation.

WIS (%)													
Time (h)	MAT.IB.100	MAT.IB.101	MAT.IB.102	MAT.IB.103	MAT.IB.104	MAT.IB.105	MAT.IB.106	MAT.IB.107	MAT.IB.108	MAT.IB.109	MAT.IB.110	MAT.IB.111	MAT.IB.112
0	9.85 ± 0.01	9.26 ± 0.01	10.2 ± 0.00	6.86 ± 0.01	9.77 ± 0.01	10.0 ± 0.00	8.74 ± 0.02	9.15 ± 0.01	9.12 ± 0.01	10.4 ± 0.01	10.0 ± 0.00	11.6 ± 0.01	10.5 ± 0.01
72	8.71 ± 0.00	9.80 ± 0.00	9.33 ± 0.00	13.4 ± 0.00	11.3 ± 0.00	11.6 ± 0.01	11.1 ± 0.00	9.51 ± 0.00	9.63 ± 0.01	9.41 ± 0.01	11.5 ± 0.00	10.7 ± 0.00	11.5 ± 0.00

E

Appendix 5

E.1 Cell viability

The cell concentration were calculated using Equation 3.2 (Table E.1). Materials MAT.IB.100 to MAT.IB.102 as well as one of the triplicates of MAT.IB.103, the samples were diluted to a 10^{-4} dilution before cell counting for all time points except 72 h instead of a 10^{-1} dilution. The CFUs were calculated using Equation 3.1. Materials MAT.IB.100 to MAT.IB.102 as well as one of the triplicates of MAT.IB.103, the samples were diluted to a 10^{-1} dilution before streaking for all time points except 72 h instead of a 10^{-4} dilution. The growth rate was calculated using equations 3.3-3.7.

Table E.1: Cell concentration, CFU and growth rate of each material sample at the different time points with standard deviation.

Cell viability													
Time (h)	MAT.IB.100	MAT.IB.101	MAT.IB.102	MAT.IB.103	MAT.IB.104	MAT.IB.105	MAT.IB.106	MAT.IB.107	MAT.IB.108	MAT.IB.109	MAT.IB.110	MAT.IB.111	MAT.IB.112
Cell concentration (Cells ml^{-1})													
0	0 ± 0	0 ± 0	0 ± 0	155733 ± 110892	262400 ± 32634	264533 ± 59370	353067 ± 72471	222933 ± 93344	292267 ± 29134	329600 ± 22627	192000 ± 187029	209067 ± 104849	272000 ± 39192
3	0 ± 0	0 ± 0	0 ± 0	146133 ± 103340	288000 ± 27527	266667 ± 34100	156800 ± 18290	180267 ± 46176	170667 ± 19610	213333 ± 98191	240000 ± 17133	201600 ± 25197	185600 ± 59867
6	0 ± 0	0 ± 0	0 ± 0	196267 ± 138781	261333 ± 40505	217600 ± 23947	126933 ± 59255	180267 ± 13151	170667 ± 14390	201600 ± 43170	217600 ± 16317	188800 ± 2613	189867 ± 47917
24	0 ± 0	0 ± 0	0 ± 0	153600 ± 108737	238933 ± 25644	199467 ± 33084	174933 ± 15964	145067 ± 71761	172800 ± 50866	232533 ± 33290	187733 ± 46028	217600 ± 30804	124800 ± 13064
33	0 ± 0	0 ± 0	0 ± 0	186667 ± 132155	262400 ± 83976	241067 ± 46470	285867 ± 59942	151467 ± 62779	121600 ± 2613	213333 ± 28661	128000 ± 9421	193067 ± 17785	135467 ± 30952
48	11 ± 15	139 ± 80	85 ± 60	235808 ± 166588	212267 ± 72518	237867 ± 47631	234667 ± 41668	161067 ± 83989	131200 ± 57303	210133 ± 24695	97067 ± 18352	128000 ± 47964	199467 ± 17000
57	5227 ± 1740	2880 ± 2041	$2240/pm453$	178560 ± 126501	283733 ± 87532	257067 ± 59942	253867 ± 37893	169600 ± 52190	125867 ± 29714	187733 ± 19259	177067 ± 6575	154667 ± 6575	157867 ± 6575
72	2240 ± 784	1173 ± 544	1813 ± 544	171093 ± 120190	195200 ± 63840	299733 ± 21756	291200 ± 29676	210133 ± 53567	179200 ± 6913	194133 ± 48904	135467 ± 29134	180267 ± 21756	142933 ± 13408
CFU (CFU ml^{-1})													
0	$TNTC \pm n.a.$	$TNTC \pm n.a.$	$TNTC \pm n.a.$	48216667 ± 5866122	30611111 ± 4899987	32822222 ± 6307276	27600000 ± 8935463	36414286 ± 4157413	36537500 ± 9641439	32037500 ± 5424928	44533333 ± 2515287	38788889 ± 5117605	37777778 ± 6235343
24	$TNTC \pm n.a.$	343889 ± 220791	$TNTC \pm n.a.$	2033333 ± 674949	0.00 ± 0.00	77778 ± 122726	0.00 ± 0.00	177778 ± 131468	0.00 ± 0.00	0.00 ± 0.00	3433333 ± 1403963	0.00 ± 0.00	0.00 ± 0.00
48	$TNTC \pm n.a.$	82333 ± 116503	$TNTC \pm n.a.$	2366667 ± 743117	0.00 ± 0.00	11111 ± 31427	0.00 ± 0.00	11111 ± 31427	0.00 ± 0.00	0.00 ± 0.00	3288889 ± 2371331	0.00 ± 0.00	0.00 ± 0.00
72	6655556 ± 1248209	22222 ± 41574	11888889 ± 1623059	2900000 ± 1240072	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8488889 ± 6399209	0.00 ± 0.00	0.00 ± 0.00
Specific growth rate (h^{-1})													
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-0.01 ± 0.02	0.03 ± 0.06	0.01 ± 0.10	-0.27 ± 0.10	-0.06 ± 0.23	-0.18 ± 0.06	-0.18 ± 0.16	0.26 ± 0.35	0.05 ± 0.26	-0.14 ± 0.16
6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.07 ± 0.05	-0.04 ± 0.07	-0.07 ± 0.04	-0.12 ± 0.22	0.01 ± 0.12	0.00 ± 0.07	0.01 ± 0.08	-0.03 ± 0.02	-0.02 ± 0.04	0.01 ± 0.17
24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.04	0.02 ± 0.01	0.05 ± 0.02	0.01 ± 0.05	-0.03 ± 0.03	-0.01 ± 0.02	-0.04 ± 0.03	-0.01 ± 0.01	0.01 ± 0.01
48	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	-0.01 ± 0.05	0.00 ± 0.03	-0.01 ± 0.03	0.00 ± 0.03	0.00 ± 0.03	0.00 ± 0.01	-0.02 ± 0.01	-0.03 ± 0.03	0.03 ± 0.02
57	0.18 ± 0.25	0.34 ± 0.17	0.22 ± 0.15	0.04 ± 0.11	0.03 ± 0.08	0.01 ± 0.05	0.01 ± 0.03	0.02 ± 0.03	0.00 ± 0.03	-0.01 ± 0.01	0.07 ± 0.02	0.03 ± 0.05	-0.03 ± 0.01
72	-0.06 ± 0.03	-0.05 ± 0.02	-0.02 ± 0.01	0.00 ± 0.01	-0.03 ± 0.04	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.03	0.03 ± 0.02	0.00 ± 0.01	-0.02 ± 0.02	0.01 ± 0.01	-0.01 ± 0.01